Regulation of IRS1/Akt insulin signaling by microRNA-128a during myogenesis

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Summary

Skeletal muscle possesses a strong ability to regenerate following injury, a fact that has been largely attributed to satellite cells. Satellite cells are skeletal muscle stem cells located beneath the basal lamina of the myofiber and are the principal cellular source of growth and regeneration in skeletal muscle. MicroRNAs (miRNAs) play key roles in modulating several cellular processes by targeting multiple mRNAs that comprise of a single or multiple signaling pathway. Several miRNAs have been shown to regulate satellite cell activity, such as miRNA-489 which functions to maintain satellite cells in a quiescent state. While muscle-specific miRNAs have been identified, many of the molecular mechanisms that regulate myogenesis that are regulated by miRNAs still remain unknown. In this study, we have shown that miR-128a is highly expressed in brain and skeletal muscle, and increases during myoblast differentiation. MiR-128a was found to regulate the target genes involved in insulin signaling, which include: Insr, Irs1, and Pik3r1 at both the mRNA and protein level. Overexpression of miR-128a in myoblasts inhibited cell proliferation by targeting IRS1. Conversely, inhibition of miR-128a induced myotube maturation and myofiber hypertrophy in vitro and in vivo. Moreover, our results demonstrate that miR-128a expression levels are negatively controlled by tumor necrosis factor-alpha (TNF-α). TNF-α promoted myoblast proliferation and myotube hypertrophy by facilitating IRS1/Akt signaling via a direct decrease of miR-128a expression in both myoblasts and myotubes. In summary, we demonstrate that miR-128a regulates myoblast proliferation and myotube hypertrophy, and provides a novel mechanism through which IRS1-dependent insulin signaling is regulated in skeletal muscle.
Introduction

Skeletal muscle has the ability to regenerate following injury or disease. Muscle regeneration is mediated primarily by mononucleated myogenic stem cells, referred to as satellite cells (Collins et al., 2005; Mauro, 1961). Satellite cells are found beneath the basal lamina of myofibers and remain in a quiescent state. Satellite cells are activated by muscle injury, exercise or disease in which they are induced to proliferate, differentiate and fuse to form multinucleated myofibers.

Duchenne muscular dystrophy (DMD) is caused by lack of dystrophin protein at the sarcolemma of muscle fibers and is characterized by progressive muscle weakness associated with continuous degeneration and regeneration of skeletal myofibers (Hoffman et al., 1987; Hoffman et al., 1988; Monaco et al., 1986). It has been reported that loss of satellite cell regenerative capacity due to continual needs for regeneration may contribute to disease progression in DMD (Luz et al., 2002; Mouly et al., 2005; Wright, 1985).

Skeletal muscle also exhibits tremendous plasticity depending on their activity, which is tightly regulated via a balance of protein synthesis and degradation. Increased protein synthesis results in muscle hypertrophy, whereas elevated protein degradation causes muscle atrophy.

Additionally, skeletal muscle function is regulated by the nervous system. Muscle atrophy is caused by the prolonged electrical inactivity including immobilization, denervation or muscle unloading. These observations indicate that the stimulation of physiological activity of muscles is an important regulator to maintain skeletal muscle mass (Jackman and Kandarian, 2004).

Recently, a number of studies have proposed novel aspects of muscle gene regulation in which small non-coding RNAs, known as microRNAs (miRNAs), play fundamental roles in various
biological processes including development, cell proliferation and differentiation (Bartel, 2004; Krol et al., 2010; Stefani and Slack, 2008). MiRNAs are from 17 to 24 nucleotides in length and are known to interact with 3’-untranslated regions (3’ UTR) of target mRNAs, resulting in the repression of mRNA translation and/or stability (Bartel, 2004). Since miRNAs can repress multiple target mRNAs, miRNA expression patterns and their predicted target mRNAs might represent a novel regulatory network (Eisenberg et al., 2007; Greco et al., 2009; Williams et al., 2009). MiRNAs are involved in the process of regeneration and hypertrophy/atrophy in skeletal muscle. In particular, miR-1, -133 and -206, which have been identified as muscle-enriched mRNAs, are known to play central regulatory roles in myogenesis (Chen et al., 2006; Kim et al., 2006; Rao et al., 2006). Additional miRNAs have been reported to regulate myoblast proliferation or differentiation which include miR-24, -26a, -27b, -125b, -148a, -181, and -214 (Crist et al., 2009; Ge et al., 2011; Liu et al., 2010; Naguibneva et al., 2006; Sun et al., 2008; Wong and Tellam, 2008; Zhang et al., 2012). Another microRNA, miR-489, is highly expressed in quiescent satellite cells and maintains satellite cell quiescence by suppressing expression of the oncogene Dek (Cheung et al., 2012). Another class of muscle-enriched microRNAs, or myomiRs, which consist of miR-23b, -195 and -208 are involved in muscle hypertrophy/atrophy (van Rooij et al., 2006; van Rooij et al., 2007; Wada et al., 2011). These observations suggest that miRNAs regulate several stages of myogenesis by repressing many target genes and critical time points during development and disease.

Here, we investigated the function of miR-128a in skeletal muscle during several myogenic processes. MiR-128a is expressed in hematopoietic stem cells and has been shown to play an
important role in cell lineage determination in neuronal stem cell, as well as muscle side
population cells (Georgantas et al., 2007; Motohashi et al., 2012; Smirnova et al., 2005). In
addition, it has been reported that cell proliferation in several types of tumor cells is regulated by
miR-128a expression (Godlewski et al., 2008; Venkataraman et al., 2010). However, despite its
enriched expression in skeletal muscle (Lee et al., 2008) the function of miR-128a in this tissue
has not been fully elucidated. Using biological pathway analysis programs, we identified
several predicted miR-128a targets in the insulin signaling pathway including insulin receptor
(Insr), insulin receptor substrate 1(Insr1) and phosphatidylinositol 3-kinases regulatory 1 (Pik3r1;
also referred to as p85α). We show that miR-128a directly targets the 3’ UTR region of these
insulin signaling genes to negatively regulate their expression levels. We also demonstrate that
miR-128a modulates myoblast proliferation and myotube formation in vitro and regulates muscle
hypertrophy in vivo. Furthermore, we identify the proinflammatory cytokine tumor necrosis
factor-alpha (TNF-α), as an enhancer of activation of IRS1/Akt signaling pathway via a decrease
of miR-128a expression in myoblasts and myotubes. Our observations highlight the role of
miR-128a in skeletal muscle and suggest that regulation of muscle mass may be possible through
modulation of miR-128a activity.

Results

Expression of miR-128a in skeletal muscle and myogenic cells

To identify the expression profile of miR-128a in adult tissues, we first performed qPCR analysis
of miR-128a levels in various adult mouse tissues. MiR-128a expression was the highest in
brain and skeletal muscle, which is consistent with previously published findings (Fig. 1A) (Lee et al., 2008). Previously, it was shown that miR-128a was increased in expression during myoblast differentiation (Chen et al., 2010; Sun et al., 2010). To confirm this, miR-128a expression was analyzed in quiescent satellite cells analyzed immediately after isolation by fluorescence-activated cell sorting (FACS), in myoblasts derived from satellite cells cultured in growth medium for 4 days, and in myotubes, which were derived from myoblasts maintained in differentiation medium for 5 days. Mononuclear cells from uninjured fore- and hind-limb muscle of adult mice were purified by FACS as CD45 negative CD31 negative and Vcam1 positive cells (Supplementary Fig. 1A). Expression of Pax7 and MyoD were assessed to confirm cell purity and further validate the isolated myogenic population as satellite cells (Supplementary Fig. 1B) (Fukada et al., 2007). The level of miR-128a expression increased during myogenic differentiation from quiescent satellite cells to myotubes (Fig. 1B) which is in agreement with previous reports (Chen et al., 2010; Sun et al., 2010). We then investigated miR-128a expression during skeletal muscle regeneration in mouse tibialis anterior (TA) muscle following a cardiotoxin-induced (CTX) injury. MiR-128a expression levels were analyzed by qPCR during a time course of 0 (uninjured), 1, 3, 5, 7 and 14 days post cardiotoxin-induced muscle injuries. MiR-128a expression decreased immediately following CTX-injury (Fig. 1C). Interestingly, during the myoblast proliferative phase 3 days following CTX muscle injury, miR-128a levels were slightly increased (Fig. 1C). MiR-128a expression remained at low levels until day 14 when the levels began to increase (Fig. 1C).
To identify potential targets of miR-128a in muscle, we utilized the computational prediction program TargetScan (http://www.targetscan.org/) which yielded >500 predicted target genes for miR-128a. The molecular functional clustering of the predicted target genes of miR-128a was determined by the use of Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation analyses in the Database for Annotation, Visualization and Integrated Discovery tool (DAVID: http://david.abcc.ncifcrf.gov) (Table 1 and Supplementary Table 2) (Dennis et al., 2003; Huang da et al., 2009). GO term annotation analysis of miR-128a target genes showed that over 60% of predicted target genes were classified directly as “cellular process”, while other GO terms (biological regulation and developmental process) were enriched as well (Supplementary Table 2). KEGG pathway annotation analysis was then performed on miR-128a predicted target genes within the “Cellular process” GO category. This analysis revealed that members of the insulin signaling pathway, which includes Pde3a, Pde3b, Insr, Irs1, Pik3r1, Pdpk1, Tsc1 and Rps6kb1, was the most enriched target pathway (Table 1) that might be directly regulated by miR-128a.

MiR-128a targets several genes involved in insulin signaling pathway

To determine if miR-128a could be directly involved in suppressing the predicted target genes in insulin signaling pathway, the 3’ UTR of each gene was cloned into a luciferase reporter construct and transfected into HEK293T cells along with plasmid constructs that overexpressed

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18  To determine if miR-128a could be directly involved in suppressing the predicted target genes in insulin signaling pathway, the 3’ UTR of each gene was cloned into a luciferase reporter construct and transfected into HEK293T cells along with plasmid constructs that overexpressed
either mouse miR-128a or scrambled-miR controls (CON). The 3’ UTR in genes related to insulin signaling (IRS1, INSR, PIK3r1) was predicted to have a single miR-128a target site with complete complementary sequence in the seed region that is comprised of 6-8 nucleotides essential for miRNA/mRNA binding (Fig. 2A). MiR-128a overexpression dramatically inhibited the reporter activity of the Irs1, Insr, and Pik3r1 luciferase reporter constructs (Fig. 2B). Importantly, after miR-128a predicted target site in the 3’ UTR was mutated, the mutated reporter construct failed to activate the luciferase reporter following miR-128a overexpression (Fig. 2B). These findings suggest that predicted target site of each gene is directly affected by miR-128a expression levels and that miR-128a targeted the 3’ UTR of these three insulin pathway related genes.

MiR-128a regulates myoblast cell proliferation by targeting IRS1

To address the potential role of miR-128a during myoblast proliferation, we induced or inhibited miR-128a expression in primary mouse myoblasts using lentiviral vectors containing green fluorescent protein (GFP) fluorescent reporters. Three days after culture of primary myoblasts, the viruses were added to the culture medium. Seventy-two hours after infection, infected myoblasts were purified by FACS based on GFP expression and subjected to cell proliferation assays. MiR-128a expression was approximately forty-fold higher in cells over-expressing miR-128a compared to control cells after lentivirus infection, whereas in miR-128a silenced cells, miR-128a expression was not at detectable levels by qPCR (data not shown). As shown in Fig. 3A, overexpression of miR-128a attenuated myoblast proliferation, whereas miR-128a inhibition
in myoblasts promoted cellular proliferation. Additionally, when the cells were stained with phospho-histone-H3 antibody, a marker for mitotic cells, overexpression of miR-128a reduced myoblast proliferation which is indicated by significantly decreased numbers of phospho-histone-H3 positive cells in the miR-128a overexpressing myoblasts as compared to scrambled-miR control (CON) cells (Fig. 3B). Conversely, we found that inhibition of miR-128a promoted myoblast proliferation, as shown by the increase in the number of phospho-histone H3 cells as compared to scrambled-miR control cells (Fig. 3B).

Insulin and insulin-like growth factors (IGFs) are well known to stimulate proliferation and differentiation of myoblasts (Engert et al., 1996), in addition to increase protein synthesis (Fryburg, 1994). IRS1/PIK3/Akt signaling is the major pathway controlled by IGF1 (Walker et al., 2010). Binding of IGF1 with the insulin receptor (INSR) induces tyrosine phosphorylation of Insulin receptor substrate (IRS) 1, which in turn activates phosphatidylinositol 3-kinase (PIK3) and its downstream target, Akt. Our observations that miR-128a targets genes related to insulin signaling and represses myoblast proliferation lead us to hypothesize that miR-128a directly restricted INSR or IRS1 expression which would result in a subsequent activation of Akt in myoblasts. To test this hypothesis, miR-128a was overexpressed in primary mouse myoblasts cultured in growth medium, and followed by analysis of the protein levels of genes related to insulin signaling and Akt-phosphorylation. IRS1 protein was reduced in proliferating myoblasts that overexpressed miR-128a, while protein levels of INSR and PI3Kr1 did not show significant changes in expression levels (Fig. 3C, D). Phosphorylated Akt levels were significantly
decreased in miR-128a overexpressing myoblasts (Fig. 3C, D). These results indicated that
overexpression of miR-128a in myoblasts attenuated cell proliferation by suppressing the levels
of IRS1 and phosphorylated-Akt. At the same time, mRNA expression of myogenic
transcriptional factors (Myf5, Pax7, MyoD and Myogenin) did not significantly change by
overexpression or suppression of miR-128a (Fig. 3E). As previous studies indicated, the Akt
pathway regulates cell cycle and cellular proliferation by modulating the expression levels of
critical cell cycle regulatory genes, such as p21, independent of MRF-gene expression (Lawlor
and Rotwein, 2000). Collectively, these studies suggest that miR-128a might regulate cell
proliferation via expression of Akt signaling pathway components, not via MyoD or Myogenin
expression.

MiR-128 inhibition does not affect myoblast differentiation, but promotes IRS1/Akt signaling
and myocyte hypertrophy
To study the function of miR-128a during myoblasts differentiation, we overexpressed or
inhibited miR-128a in primary mouse myoblasts and then induced them to differentiate for 3 days.
Differentiated myotubes were stained with myosin heavy chain (MyHC) antibody
(Supplementary Fig. 2), and the percentage of nuclei in MyHC-positive cells (fusion index) were
quantified. We observed no significant differences in the overall fusion indexes among each
group (Fig. 4A). Interestingly, the number of myotubes with 10 or more nuclei was
significantly increased when miR-128a was reduced as compared with cultures where miR-128a
was overexpressed (Fig. 4B). To determine what effect miR-128a expression levels had on
myogenic factors during differentiation, the expression levels of myogenic genes (Myf5, Pax7, MyoD and Myogenin) were analyzed by qPCR. Both Pax7 and MyoD, which are expressed during myoblast proliferation and early phase of myoblast differentiation and which are abolished in late stage of myoblast differentiation, were significantly decreased in anti miR-128 myotubes compared to control myotubes. No change in Myogenin expression was detected in the miR-128a inhibited myotubes (Fig. 4C). These results indicate that inhibition of miR-128a may promote the progression of myotube maturation instead of myoblast fusion.

To assess the protein levels of miR-128a targets and Akt signaling components during myoblast differentiation, C2C12 myoblasts transfected with either miR-128a, antimiR-128a, or control viruses were induced to differentiate in differentiation medium. One day after differentiation, the protein levels of IRS1 and phosphorylated-Akt were significantly higher in Anti miR-128 myotubes than in control myotubes (Fig. 4D, E). Three days after differentiation, the level of IRS1 was still higher in Anti miR-128 myotubes than in control myotubes, while phosphorylated-Akt level was slightly lower in Anti miR-128 myotubes relative to control myotubes (Fig. 4D, E). Five days after the start of differentiation, IRS1 and phospho-Akt levels could no longer be detected in Anti miR-128 myotubes, even though INSR and PIK3 expression levels could still be detected in both control and Anti miR-128 myotubes (Fig. 4F). Moreover, to determine if Anti miR-128 induces myotube hypertrophy, we measured C2C12 myotube size, since IGF1-mediated activated Akt has been proposed to increase the size of myotubes (Rommel et al., 2001). The diameter of myotubes where miR-128 was inhibited was measured 5 days...
after differentiation, and quantified as shown in Fig. 4G and Supplementary Fig. 3. Inhibition of miR-128a resulted in significantly larger myotubes (36.1 ± 0.75 µm; n=3) when compared to controls (29.2 ± 0.65 µm; n=3) (Fig. 4G and Supplementary Fig. 3). Collectively, these studies suggested that inhibition of miR-128a may promote myotube maturation or hypertrophy.

Inhibition of miR-128 by minicircle vectors induced muscle hypertrophy in vivo

To explore the function of miR-128a in skeletal muscle in vivo, non-viral minicircle vectors containing antisense miR-128 (Anti miR-128) or scramble miRNA control (CON) were introduced into wild-type mice via tail vein (Supplementary Fig. 4). Minicircle vectors lack a bacterial backbone sequence and the antibiotic selection marker, which can provide higher transfection efficiency, less immunological rejection and longer transgene expression (Chen et al., 2003). We performed intravenous injection with 50 µg of minicircle vectors carrying Anti miR-128 or scrambled miRNA into 4 week old C57BL/6 wild type mice twice a week for 4 weeks. Total body and individual mass of muscles was examined following treatment (gastrocnemius, quadriceps and tibialis anterior). Anti miR-128 treatment resulted in significant gain of entire body weight and muscle volume, compared to control miR treated mice (Fig. 5A and B). At the same time, the transcriptional levels of miR-128a target containing genes, Irs1, Insr and Pik3r1 were slightly elevated by Anti miR-128 compared to scramble control miR, but there were no significant differences between control and anti miR-128 injected muscle (Fig. 5C). Consistently, anti miR-128 inhibition significantly induced myofiber hypertrophy, as shown by the increased distribution of myofiber diameter (Fig. 5D). To examine a potential relationship
between muscle hypertrophy induced by Anti miR-128 and muscle fiber type, we compared myosin heavy chain (MyHC) isoform expression by qPCR in gastrocnemius muscles. Although there were no significant differences, Myh7 (MyHC type I) expression was slightly decreased for the mice treated with Anti miR-128, while the expression levels of Myh2 (MyHC type IIa), Myh1 (MyHC type IIX) and Myh4 (MyHC type IIb) were slightly elevated in muscle where miR-128a was inhibited as compared to control injections. However, there were no significant differences between control and anti miR-128 injected muscles (Fig. 5E).

TNF-α is a regulator of miR-128a and up-regulates IRS1/Akt signaling pathway by suppressing miR-128a

MiR-128a expression is decreased in early phase of skeletal muscle regeneration as shown in Fig. 1D and previously published results (Chen et al., 2010), suggesting that the factors or cytokines secreted by inflammatory cells such as neutrophils and macrophages may regulate miR-128a expression. TNF-α (tumor necrosis factor-alpha) is a major pro-inflammatory cytokine that plays an important role in muscle regeneration by promoting myoblast proliferation and differentiation (Alter et al., 2008; Chen et al., 2007; Li, 2003; Li and Schwartz, 2001; Warren et al., 2002). TNF-α expression is up-regulated immediately following CTX injection in mouse muscles (Chen et al., 2007). Furthermore, TNF-α is also known to regulate miR-128a by targeting the adenosine 2B receptor (A2BAR) in epithelial cells (Kolachala et al., 2010), which in turn regulates insulin signaling and myoblast proliferation (Alter et al., 2008; Li, 2003; Yamasaki
et al., 1996). We first examined the effects of TNF-α on miR-128a expression in myoblasts.

Primary myoblasts and C2C12 myoblasts cultured with growth medium were treated with mouse

TNF-α recombinant protein (20 ng / ml), and then analyzed miR-128a expression by qPCR.

TNF-α treated samples showed a decrease in miR128a expression levels during the proliferation

phase of primary myoblasts ($p = 0.054$) and C2C12 myoblast ($p < 0.01$) (Fig. 6A). To further

investigate the link between TNF-α and the regulation of miR-128a expression, we assessed

whether the decline of miR-128a expression levels observed after TNF-α treatment might

stimulate cell proliferation by promoting IRS1 and Akt activation. TNF-α treatment

significantly increased myoblast proliferation, as indicated by the increased number of phospho

histone-H3 positive cells in TNF-α treated myoblasts when compared to non-treated myoblasts

(Fig. 6B). Protein expression of IRS1 and phosphorylated-Akt was elevated after TNF-α

treatment (Fig. 6C), consistent with previous reports that TNF-α promotes glucose uptake and

cell proliferation (Li, 2003; Yamasaki et al., 1996). These observations imply that miR-128a

and its target genes might be regulated under the control of TNF-α during myoblast proliferation.

Next we examined the effects of TNF-α treatment in C2C12 myotubes to determine if TNF-α

decreases miR-128a expression and promotes increase of IRS1/Akt signaling. According to

previous reports, TNF-α inhibits myoblast fusion but at the same time also increases protein

amounts in myotubes mediated by PI3K-Akt signaling pathway (Alter et al., 2008; Plaisance et

al., 2008). Treatment with TNF-α decreased miR-128a expression in C2C12 myotubes, and

increased protein levels of IRS1, PI3Kr1 and phosphorylated-Akt (Fig. 6D and F). Although
these observations support the fact that TNF-α regulates miR-128a expression and IRS1/Akt signaling, it did not address whether TNF-α directly or indirectly regulates IRS1/Akt signaling during myogenesis. To clarify this concept, C2C12 myoblasts overexpressing miR-128a from a lentiviral vector were induced in differentiation medium for 5 days, followed by 24 hour-treatment with/without TNF-α in serum-free medium, after which expression of miR-128a target genes were evaluated. As shown in Fig. 6G and H, miR-128a expression levels as well as the protein levels of IRS1, PIK3r1 and phosphorylated-Akt were not altered in TNF-α-treated myotubes in which miR-128a is overexpressed. This finding suggested that increase of IRS1, PIK3r1 and phosphorylated-Akt levels required the subsequent down-regulation of miR-128a expression levels by TNF-α. Lastly, C2C12 myotubes were cultured with/without TNF-α to determine if the reduction of miR-128a levels caused by TNF-α treatment would induce the changes of IRS1 and pAkt levels and subsequent myotube hypertrophy, as observed in miR-128 inhibited myotubes (Fig. 4D-G). C2C12 myoblasts were induced into myotube with differentiation medium for 2 days, and then differentiated myotubes were cultured under the differentiation conditions with/without TNF-α (Fig. 6I), because TNF-α is known to hinder the early phase of myoblast differentiation (Alter et al., 2008; Coletti et al., 2002). The inhibition of myoblast fusion and the reduction of miR-128a was observed with the presence of TNF-α (Fig. 6A and Supplementary Fig. 5A). When miR-128a overexpressing myoblasts were treated with TNF-α, the inhibition of myoblast fusion was observed while miR-128a expression levels were not altered (Fig. 6G and Supplementary Fig. 5B). These data suggest that TNF-α inhibits
myoblast fusion regardless of miR-128a expression levels. TNF-α treatment resulted in
significantly thicker myotubes than control myotubes three days following initial exposure to
TNF-α or mock controls (Fig. 6I). Furthermore, to assess the protein levels of miR-128a targets
and Akt signaling after treatment of TNF-α, myotubes underwent western blot analysis. One
day after TNF-α treatment, the levels of IRS1 and phosphorylated-Akt were higher in TNF-α
treated myotubes than in mock control myotubes (Fig. 6J). Five days after the initiation of
TNF-α treatment, IRS1 levels in TNF-α treated myotubes had declined to the same level of that
observed in control myotubes. Furthermore, phospho-Akt levels in TNF-α treated myotubes
were lower than mock controls even though INSR and PIK3 expression levels remained same in
both control and TNF-α treated myotubes (Fig. 6J). When TNF-α treatment was performed in
miR-128a overexpressing myotubes, no significant changes in myotube size was observed
(Supplementary Fig. 5C). These observations indicated a strong regulation of genes related to
the insulin-signaling pathway by miR-128a and TNF-α during myogenesis. Additionally, to
evaluate the effect of insulin addition and to determine if IRS1 stimulation by TNF-α is sensitive
to insulin, C2C12 myoblasts were differentiated into myotubes for 5 days, then treated with
TNF-α and/or insulin. Our results indicated that increased IRS1 levels by TNF-α treatment are
subsequently decreased by the addition of exogenous insulin. This finding suggested that the
sensitivity of IRS1 to insulin in myotubes was increased after treatment of TNF-a
(Supplementary Fig. 6). Collectively, these observations suggest that TNF-a might promote
myotube hypertrophy caused by reduction of miR-128a levels. Furthermore, the successive
Discussion

MiR-128a regulates myoblast proliferation and differentiation through IRS1/Akt signaling

Our study has identified miR-128a as a novel regulator of insulin signaling and characterized its function in skeletal muscle. Recent studies have shown that miR-128a expression is reduced in several tumor cell lines compared to normal cells through suppression of cell proliferation by targeting E2F3, Bmi-1 or p70S6K1 (Godlewski et al., 2008; Shi et al., 2012; Zhang et al., 2009). Our findings have highlighted miR-128a function in myogenesis, and identified novel muscle-enriched target gene, IRS1. Small RNAi-based gene silencing experiments revealed that insulin signaling pathways are dependent on Irs1 and are required for myotube formation and glucose uptake through the phosphorylation of Akt (Bouzakri et al., 2006). In addition, phosphorylated-Akt promotes myoblast proliferation in cooperation with mTOR or S6K1 (Naguibneva et al., 2006), suggesting that IRS1 is a key factor to induce myoblast proliferation and myotube hypertrophy by increasing phospho-Akt levels, and thus miR-128a inhibited myoblasts might be resulting from the increase of IRS1/Akt phosphorylation. Additionally, we demonstrated that miR-128a expression levels increased during the myogenic differentiation of primary myoblasts (Fig. 1C). These observations suggest that miR-128a might also act as a negative regulator of insulin signaling in myotubes. MiR-128a has
additional, non-insulin signaling pathway predicted target genes which include: *Foxo1, Gsk3b* and *Pten* (Table 1). These three factors are also known to negatively regulate the phosphorylation of Akt. *Foxo1* (forkhead transcription factor O 1) and GSK (glycogen synthase kinase 3 beta) are downstream targets of PI3K/AKT signaling in muscle (Rommel et al., 2001; Sandri et al., 2004). Activation of *Foxo1* caused by PIK3/Akt phosphorylation induces the ubiquitin ligase, Atrogin-1, resulting in muscle atrophy (Sandri et al., 2004), and activation of GSK3β inhibits protein synthesis by inhibiting the translation initiation factor eIF2B (Rommel et al., 2001). In addition, miR-128a was recently reported to suppress Akt activity by targeting PTEN in pituitary tumors (Palumbo et al., 2012). Moreover, another miRNA, let-7, was also recently shown to regulate the insulin signaling pathway by targeting multiple genes including *Igfr1, Insr* and *Irs2* in skeletal muscle (Zhu et al., 2011). Thus, it is likely that insulin signaling is regulated by several miRNAs in addition to miR-128a, which may also be IRS1-dependent and induce the phosphorylation of Akt during myotube hypertrophy.

**TNF-α as a positive regulator of insulin signaling through repression of miR-128a**

There is increasing evidence that TNF-α, a major pro-inflammatory cytokine produced by activated macrophages, has an important role in muscle regeneration by promoting myoblast proliferation and differentiation (Alter et al., 2008; Chen et al., 2007; Li, 2003; Li and Schwartz, 2001; Warren et al., 2002). TNF-α has also been shown to induce muscle wasting through the inhibition of protein synthesis, selective proteolysis, and the activation of NF-κB and
caspase-dependent pathways (Coletti et al., 2002; Li and Reid, 2000). Prior studies have demonstrated that TNF-α-deficient \textit{mdx} dystrophic mice showed decreased muscle mass and increased dystrophic disease progression, further implying that TNF-α expression may be essential for normal skeletal muscle regeneration (Spencer et al., 2000). Our findings suggest that TNF-α promotes IRS1/Akt signaling through the suppression of miR-128a expression in myoblasts and myotubes. It has been reported that TNF-α treated myoblasts increased total DNA content by accelerating cell cycle progression in a dose-dependent manner, whereas the high concentrations of TNF-α induced cell death (Li, 2003; Li and Reid, 2000). Additionally, TNF-α is thought to be secreted from myoblasts and myotubes, and to have a role as an autocrine factor in the activation of p38 (Chen et al., 2007). These observations and our results indicate that TNF-α might regulate insulin signaling through miR-128a expression.

MiRNAs are important contributors to mechanisms of disease and offer the potential to generate novel target therapies (Eisenberg et al., 2009; Williams et al., 2009). Several miRNAs have been implicated in disease progression and pathology, including muscular dystrophy (Cacchiarelli et al., 2010; Eisenberg et al., 2007). Our results suggest that inhibition of miR-128a might promote muscle regeneration and hypertrophy by inhibiting the expression of insulin signaling factors. Our findings demonstrate that miR-128a is likely to have an important role in cell growth and proliferation of skeletal muscle, and understanding its function can offer new insight into mechanisms of muscle regeneration, further opening up the potential for designing novel target therapies for muscle diseases.
Materials and Methods

Animals and cardiotoxin (CTX) injection

Animals were housed in the Animal Resources Children’s Hospital (ARCH) and all procedures were approved by the Children’s Hospital Boston Animal Facilities/IACUC protocols. C57BL/6 mice were originally purchased from Jackson Laboratory (Bar Harbor, ME), housed and bred according to standard procedures.

To induce muscle regeneration, cardiotoxin (CTX, 10 µM; Sigma, St. Louis, MO) was injected into the tibialis anterior (TA) muscle. CTX-injected TA muscles were harvested 1, 3, 7, and 14 days after injection, while the contra-lateral leg was used as day 0 uninjured control.

Isolation of Muscle Satellite Cells

Mononuclear cells were prepared from fore and hind limb muscles of 8- to 12-week-old female C57BL/6 mice. In brief, muscles were digested with 1.2 U/ml neutral protease/dispsase and 5 mg/ml collagenase IV (Worthington Biochemical, Lakewood, NJ) for 60 minutes at 37°C. After elimination of RBCs by RBC lysis solution (Qiagen, Hilden, Germany), cells were resuspended with 2% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) in PBS, and incubated on ice for 30 minutes in the presence of PE-conjugated anti-CD31 antibody (1:200, clone 390; BD Pharmingen, Franklin Lakes, NJ), FITC-conjugated anti-CD45 (1:200, clone 30-F11; BD Pharmingen), and APC-conjugated Vcam1 (1:200, clone 429; BioLegend, San Diego, CA). Propidium iodide was added to samples at a concentration of 2 µg/ml to exclude dead cells.
Cells were detected in a FACS Aria flow cytometer (BD Bioscience, Franklin Lakes, NJ).

**Cell cultures**

Satellite cells from mouse muscle were cultured in growth medium consisting of high-glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM; cellgro, Manassas, VA) with 20% FBS (Atlanta Biological), 2.5 ng/ml basic fibroblast growth factor (Invitrogen), and penicillin (100 U/ml)-streptomycin (100 mg/ml) (Gibco-BRL, Gaithersburg, MD) on culture dishes coated with Matrigel (BD Biosciences). For myotube formation, the medium was replaced to DMEM supplemented with 5% horse serum and penicillin-streptomycin to promote myoblast differentiation (differentiation medium; DM). TNF-α was purchased from R&D Systems (Minneapolis, MO), dissolved with 1% BSA in PBS, and then supplemented to the culture medium (20 ng/ml).

**RNA extraction, TaqMan miRNA expression assay and SYBR green based quantitative real-time PCR**

Total RNAs containing miRNA were extracted from satellite cells, myoblasts and myotubes using a miRNeasy RNA isolation kit (Qiagen). TaqMan microRNA expression assay was performed with 50 ng of total RNA by using TaqMan microRNA reverse transcription kit and TaqMan microRNA kit (Applied Biosystems, Foster City, CA, USA) according to the manufacture’s instructions. For gene expression analysis, first strand cDNA was produced using a QuantiTect Reverse Transcription Kit (Qiagen), and synthesized cDNA was mixed with
SYBR Green PCR Master Mix. Specific primers used for PCR are listed in Supplementary Table 1. The expression levels of miRNA and mRNA were quantified on an ABI 7900HT real-time PCR machine (Applied Biosystems) following the manufacturer’s instructions. MiRNA expression was normalized against the expression of U6, and individual mRNA expression was normalized against the expression of 18s rRNA. mRNA levels were quantified by comparative Ct (ΔΔCt) method (Schmittgen and Livak, 2008).

Functional and pathway analysis of microRNA predicted target genes

To identify the predicted miR-128a target genes, TargetScan (http://www.targetscan.org/) was used. To investigate the molecular function of the predicted target genes, biological pathway analysis of a large gene list was carried out to obtain gene lists on the basis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases in the Database for Annotation, Visualization and Integrated Discovery tool (DAVID: http://david.abcc.ncifcrf.gov) (Dennis et al., 2003; Huang da et al., 2009). P-values for all of the different genes in GO terms and KEGG pathways were calculated by DAVID, and the false discovery rate (FDR) were calculated to correct the enrichment P-value based on Benjamin-Hochberg multiple testing correction method; the significance was defined as $P < 0.01$ and FDR $< 0.05$.

Luciferase reporter assay

The 3’ UTR of mouse Irs1, Insr and Pik3r1 genes were amplified by PCR and cloned into a modified version of the pGL2Basic vector (containing a novel multi-cloning site between the
luciferase open-reading frame and the SV40 polyA signal; gift from Dr. J.A. Kreidberg) (Alexander et al., 2011) using the sequence-specific primers listed in Supplementary Table 1. The predicted miR-128a binding sites located in the 3’ UTR were mutated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), and the primers used in this experiment were listed in Supplementary Table 1. For luciferase reporter assay, 3x10^4 HEK293T cells were initially plated in a 48-well plate 24 hours prior to transfection. The cells were transfected using Lipofectamine 2000 (Invitrogen) with 30 ng of reporter construct and 100 ng of miRNA over-expression plasmids or scrambled miRNA controls (Origene, Rockville, MD). Luciferase activity was measured 48 hours after transfection with the Dual Reporter Assay System (Promega, Madison, WI) on a single-tube luminometer (Berthold Technologies, Bad Wildbad, Germany). Transfections were performed in triplicate and the individual experiments were repeated three times.

**Lentiviral miRNA overexpression and inhibition**

Lentivirus-based expression plasmids containing green fluorescent protein (GFP) that overexpress either pre-miR-128a or antimiR-128 or miRNA control, were purchased from System Biosciences (Mountain View, CA). Lentiviral vectors, along with packaging plasmids (MDL/RRE, Rev, and VSV-G) were transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen). Three days after transfection, viral supernatants were collected and filtered through 45 micron filters (VWR, West Chester, PA), mixed with Lenti-X lentivirus concentrator (Clontech, Palo Alto, CA), and incubated overnight at 4°C. The following day, the virus
co-precipitate was concentrated by centrifugation at 1,500 x g for 60 minutes at 4°C. Viral pellets were re-suspended in PBS. To overexpress or inhibit miRNA expression, the viruses were added to myoblasts in culture. Seventy-two hours after induction, miR-128a overexpressed or inhibited myoblasts were collected based on GFP expression via flow cytometry (BD Biosciences).

Western blotting

Proteins from cultured cells were extracted with RIPA buffer (Sigma) supplemented with Complete Mini protease inhibitor cocktail (Roche, Meylan, France) and PhosStop phosphatase inhibitor (Roche). Proteins (30 µg per lane) were separated by 4-20% gradient Tris-glycine gels (Invitrogen), and transferred to polyvinylidene difluoride membrane (Invitrogen) using 15 V for 1 hour. Following transfer, the membranes were blocked with 5% nonfat milk in Tris-buffered saline-Tween 20 (TBS-T) for 1 hour at room temperature, before being incubated overnight at 4°C with primary antibodies diluted with 5% bovine serum albumin (BSA; Sigma) in TBS-T. The following antibodies were used for immunoblotting; anti-IRS1, anti-INSR, anti-PDK1, anti-phospho Akt (Ser 473), anti-Akt (Cell Signaling Technology, Danvers, MA), anti-PI3Kr1 (Abcam, Cambridge, MA), p21, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). After serial washes with TBS-T, the membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) in TBS-T containing 5% milk for 1 hour at room temperature. The signals obtained from the western blot analysis were quantified with the ImageJ program (NIH).
**Immunocytochemical analysis**

Myoblasts and myotubes were cultured on 8-well Lab-Tek Chamber Slides (Nunc, Rochester, NY, USA). Cultured cells were fixed in 4% paraformaldehyde (PFA) for 5 min, stained with anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY) or mouse anti-Myosin Heavy Chain (clone: MF20; Developmental Studies Hybridoma Bank, Iowa City, IA) at 4°C overnight and then incubated with the secondary antibody conjugated with Alexa 488 or Alexa 568 (Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). The fusion indices were calculated as the percentage of nuclei in Myosin Heavy Chain-positive myocyte or myotubes per total nuclei in the dish.

**Preparation of minicircle vectors and intravenous injection**

Minicircle producer plasmid vector was purchased from System Biosciences. It contained an EF1a promoter and an H1-promoter with scramble miRNAs (CON) or antisense miR-128 (Anti miR-128) located between attB and attP sites of the parental plasmid. Minicircle DNA vectors were generated by site-specific intramolecular recombination between attB and attP mediated by PhilC31 integrase, which allowed for the plasmid backbone to be degraded in bacteria and purified following manufacturer’s instructions. Briefly, parental plasmids were transformed with E. coli strain ZYCY10P3S2T (System biosciences) and single colonies were cultured at 37°C. Bacterial cultures were spun down at 1,500g for 15 min, resuspended with LB broth containing 1% L- (+)-arabinose (System biosciences) and then cultured at 30°C for 16 hours. Minicircle
vectors were extracted using PureLink™ HiPure Plasmid Purification Kit (Invitrogen).

Prepared 50 μg of minicircle vectors carrying Anti miR-128 or scramble miRNA (CON) were injected into C57B6 wild type mice via tail vein using 29-gauge insulin syringe (Terumo Medical Products, Somerset, NJ).

**Histological analyses**

Gastrocnemius (GA), quadriceps (QC) and tibialis anterior (TA) muscles were isolated from mice and frozen in liquid nitrogen-cooled isopentane (Sigma-Aldrich). Muscle tissues were sectioned on a cryostat into 6-μm cross sections and stained with Hematoxylin and Eosin (H&E). The maximum myotube diameter from the largest branch point was measured from at least 80 myotubes with ImageJ program (NIH).

**Statistics**

All quantitative data are represented as means ± SE. Analysis was performed between different groups using 2-tailed student's t-test and nonparametric Mann-Whitney U test. A probability of less than 5% (P < 0.05) was considered statistically significant. Chi-square test was performed to assess significant differences in frequency distribution of myofiber diameter following anti miR-128 treatment.

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Footnotes

The authors declare no competing financial interests.

Author contributions: N.M: Conception and design of experiments, collection and assembly of  
data, data analysis and interpretation, manuscript writing; M.S.A, Y.M, J.A.M, G.K: Provision of  
experimental materials, data analysis and discussion of results; L.M.K: Conception and design,  
data analysis, financial support, manuscript writing and final approval of manuscript.

Figures and legends

Figure 1  Expression of miR-128a in tissues and myogenic cells

(A) Expression of miR-128a in adult mouse tissues. Quantitative PCR results show that  
miR-128a is highly expressed in brain and skeletal muscle.
(B) miR-128a expression during myogenesis. Total RNA enriched for miRNAs was extracted from mouse quiescent satellite cells, myoblasts (cultured satellite cells in growth medium for 3 days) and myotube (fused myoblasts in differentiation medium for 5 days), and analyzed by qPCR to assess miR-128 expression. The levels of miR-128a increased during myogenesis. The expression values were normalized with U6 snRNA expression, and were represented as mean±SE (n=3).

(C) miR-128a expression during skeletal muscle regeneration. Mouse tibialis anterior (TA) muscles were injured with cardiotoxin (CTX), and isolated 1, 3, 5, 7 and 14 days after CTX injection. After extraction of total RNAs, miR-128a expression was subjected to the analysis by qPCR. The levels of miR-128a declined right after CTX-injection. Values were represented as mean±SE (n=3).

Figure 2 Predicted miR-128a targets related to insulin signaling

(A) Tabulation of the predicted binding sequence of miR-128a given below the binding site in 3’ UTR of insulin signaling pathway related genes, and the mutated 3’ UTR binding sequence below. MiR-128a seed match region was highlighted in bold and mutated region was underlined.

(B) Luciferase reporter gene linked to 3’ UTR of the individual genes (WT) or 3’ UTR with mutated miR-128a binding site (Mut) was co-transfected with miR-128a (black bar) or a scrambled miRNA control expression plasmid (white bar) into 293T cells. Forty-eight hours after transfection, luciferase assay was performed. The transfection of miR-128a
overexpression vector significantly inhibited the levels of luciferase activity in cells that were transfected with 3’ UTR-pGL2 construct of individual genes, whereas it had no effect when cells are transfected with mutated 3’ UTR-pGL2 and miR-128a.

Figure 3  miR-128a negatively regulates myoblast proliferation through targeting of IRS1

(A) Myoblasts, where miR-128a is overexpressed or inhibited, were seeded the same number (10,000 cells per well in 48-well culture plate) and then counted every 2 days for 8 days. Overexpression of miR-128a repressed myoblast proliferation compared to scrambled control, whereas inhibition of miR-128 promoted proliferation of myoblasts, indicating that miR-128a is the regulator of cell proliferation in myoblasts. Values were represented as mean±SE (n=3).

(B) Immunostaining with phospho histone-H3 antibody (H3-P), which is a mitosis marker. Myoblasts cultured in growth medium were stained with H3-P antibody and counted the number of H3-P positive cells. The ratio of H3-P-positive cells per total nuclei was significantly lower in myoblasts where miR-128a is overexpressed than in control myoblasts, whereas Anti miR-128 containing myoblasts had more H3-P positive cells in comparison to control or miR-128 overexpressed myoblasts. This would indicate that myoblast proliferation is regulated by miR-128a expression levels. Values were represented as mean±SE (n=4). Scale bar = 50 μm.

(C, D) Protein levels in genes related to insulin signaling during myoblast proliferation indicated that miR-128a induced the decline of IRS1 and phospho-Akt levels, and the elevation of p21 protein levels. All quantitative results are normalized by GAPDH, and are shown as mean±SE from three independent experiments.
(E) Effect of miR-128a to the expression of myogenic transcriptional factors (Pax7, Myf5, MyoD and Myogenin) during myoblast proliferation. The levels of myogenic factors were slightly elevated by expression of the Anti miR-128 although not significantly. These transcription factors were not affected by miR-128a overexpression in primary myoblasts. The expression values were normalized with 18s rRNA expression, and were represented as mean±SE (n=3).

Figure 4  miR-128a does not affect myoblast differentiation, but inhibition of miR-128a promotes myotube hypertrophy

(A) Primary myoblasts where miR-128a is overexpressed or inhibited were induced to differentiate with DMEM medium containing 5% horse serum for 3 days. The differentiated cells were stained with Myosin heavy chain (MyHC) antibody and DAPI. The ratio of MyHC-positive cells per total nuclei (DAPI positive cells) was quantified. There were no changes among each group during myoblast differentiation. Values were represented as mean±SE (n=3).

(B) Quantification of myotube formation. Three days after differentiation, the number of nuclei per myotube was calculated and it was found that overexpression of miR-128a induced smaller myotubes with fewer nuclei relative to Anti miR-128a transduced myotubes. Values were represented as mean±SE (n=3).

(C) Evaluation of the mRNA levels of myogenic transcriptional factors (Myf5, Pax7, MyoD and Myogenin). Five days after differentiation, total RNA was extracted from myotubes, and the RNAs were subjected to analysis with qPCR. The expression of Pax7 and MyoD in myotubes
where miR-128 was suppressed was significantly reduced compared to scrambled control myotubes. The expression values were normalized with 18s rRNA expression, and were represented as mean±SE (n=3).

(D, E) C2C12 myoblasts transduced by Anti miR-128 or scrambled miR control lentivirus vectors were induced to differentiate with DMEM medium containing 5% horse serum for 1 and 3 days, and starved with serum-free medium 24 hours before extract of protein. The protein levels were explored by western blot analysis. Protein levels of IRS1 and Akt-phosphorylation were higher in Anti miR-128 transduced myotubes compared to that of control myotubes 1 day after differentiation. Three days after differentiation, the level of IRS1 was still higher in Anti miR-128 myotubes than in control myotubes although phosphorylated-Akt level was slightly lower in Anti miR-128 myotubes relative to that of control myotubes. All quantitative results are normalized by GAPDH, and are shown as mean±SE from four independent experiments.

(F) C2C12 myoblasts transduced by Anti miR-128 or scrambled control lentivirus vectors were induced to differentiate for 5 days, and starved with serum-free medium 24 hours before extract of protein. The protein levels of IRS1, INSR, PIK3r1, and Akt-phosphorylation were analyzed and quantified, indicating IRS1 and phospho-Akt levels were reduced while INSR and PIK3r1 levels were not altered in Anti miR-128 expressing myotubes. Quantitative data are normalized by GAPDH, and are shown as mean±SE (n=3).

(G) Inhibition of miR-128 induced myotube hypertrophy. C2C12 myoblasts transduced by Anti miR-128 or scrambled control lentivirus vectors were induced to differentiate for 5 days, and then stained with myosin heavy chain (MyHC) antibody (red) and DAPI (blue). Myofiber diameter
was measured and quantified. Represented data show that Anti miR-128 expressing myotubes were thicker than control expressing myotubes. Values were shown as mean±SE. Scale bar = 50 µm.

Figure 5  Inhibition of miR-128 with minicircle vector increases skeletal muscle mass and fiber size

(A) C57BL/6 wild type mice were intravenously injected with 50 µg of minicircle vectors, which have H1 promoter with Anti miR-128 or scrambled miRNA, every 4 days, and each animal analyzed for the body weight. The results indicated that the body weight of Anti miR-128 transduced mice were heavier than control mice. Values were shown as mean±SE (n=4).

(B) Thirty-five days after first injection muscles from mice where Anti miR-128 or scrambled miRNA were transduced via minicircle were isolated and each muscle was weighed (quadriceps: QC, gastrocnemius: GA and tibialis anterior: TA). The results indicate that the volume of muscles was increased in Anti miR-128 transduced mice in comparison to control mice. Values were represented as mean±SE (n=3)

(C) The expression levels of miR-128a target genes in the GA muscle from mice where Anti miR-128 or scrambled miRNA was transduced were investigated by qPCR. The expressions of miR-128a targets were elevated in Anti miR-128 transduced muscle relative to control muscle, indicating that the expression of miR-128a targets were effectively promoted by Anti miR-128 minicircle vector injection in vivo. Values were shown as mean±SE (n=4).
(D) QC muscles were isolated 35 days after first injection, sectioned and then stained with H&E. Representative images were H&E staining of QC muscle from mice where Anti miR-128 (right) or scramble miRNA (left) was transduced. The graph showed the frequency distribution of muscle fibers diameter, revealing that transduction of Anti miR-128 via minicircles promoted muscle hypertrophy in QC muscles. Values were shown as mean±SE (n=3). Scale bar = 100 µm.

(E) The expression levels of Myh7 (MyHC type I slow), Myh2 (MyHC type IIa), Myh1 (MyHC type IIx) and Myh4 (MyHC type IIb) in GA muscles were quantified by qPCR, revealing that there were no differences between Anti miR-128 or scrambled miRNA transduced mice. The expression values were normalized with 18s rRNA expression, and were represented as mean±SE (n=3).

Figure 6  TNF-α decreases miR-128a expression, and promotes myoblast proliferation through the elevation of IRS1

(A) Primary myoblasts and C2C12 myoblasts cultured in growth medium were treated with TNF-α (20 ng/ml) for 24 hours. Total RNAs containing miRNAs were extracted from TNF-α treated C2C12 myoblasts, and then subjected to analysis of the miR-128a levels by qPCR. This analysis revealed that miR-128a expression was suppressed by TNF-α. The expression values were normalized with U6 expression, and were represented as mean±SE (n=3).

(B) Primary myoblasts in growth medium were treated with TNF-α (20 ng/ml) for 3 days, stained with phospho-histone H3 (H3-P) antibody and DAPI, and the number of H3-P positive cells
calculated. The ratio of H3-P positive cells per total nuclei was significantly increased in
TNF-α-treated myoblasts than in control myoblasts, indicating that TNF-α promoted myoblast
proliferation. Values were represented as mean±SE (n=3). Scale bar = 50 µm.

(C) The protein levels of miR-128a targets (IRS1, INSR and PI3Kr1), Akt and p21 in myoblasts,
which were treated with/without TNF-α for 3 days, were evaluated by western blot analysis,
revealing that the expression levels of IRS1 and the level of Akt-phosphorylation were increased
in myoblasts treated with TNF-α in comparison to non-treated myoblasts.

(D) C2C12 myotubes, which were induced to differentiate for 5 days and then cultured with
serum-free medium supplemented with/without TNF-α (20 ng / ml) for 24 hours. Total RNAs
were extracted from TNF-α treated C2C12 myotubes, and then subjected to analysis of the
miR-128a levels by qPCR. This analysis indicated that miR-128a expression was suppressed by
TNF-α in C2C12 myotubes. The expression values were normalized with U6 expression, and
were represented as mean±SE (n=3).

(E, F) The protein levels of miR-128a targets (IRS1, INSR, PDK1 and PIK3r1) and Akt in
C2C12 myotubes, which were treated with/without TNF-α, were explored by western blot
analysis. The results of western blot analysis were quantified, suggesting that the expression
levels of IRS1, PIK3r1 and Akt-phosphorylation were increased in C2C12 myotubes treated with
TNF-α relative to non-treated myotubes. Quantitative data are normalized by GAPDH, and are
shown as mean±SE from three independent experiments.

(G) C2C12 myotubes where miR-128a was overexpressed by lentivirus vector were induced to
differentiate in differentiation medium for 5 days. miR-128a overexpressing myotubes were
cultured with serum-free medium supplemented with/without TNF-α (20 ng / ml) for 24 hours, and then was evaluated for miR-128a expression by qPCR. The results showed that miR-128a levels were not affected by TNF-α treatment in miR-128a overexpressing myotube. The expression values were normalized with U6 expression, and were represented as mean±SE (n=3).

(H) Western blot analysis to verify the protein levels of IRS1, PIK3r1 and phosphorylated Akt was performed in miR-128a overexpressing myotubes which were cultured with serum-free medium supplemented with/without TNF-α for 24 hours. The results indicated that there was no effect on miR-128a overexpressing myotubes of TNF-α treatment, revealing that the elevation of IRS1, PIK3r1 and phosphorylated-Akt levels by TNF-α treatment was mediated by the decline of miR-128a expression.

(I) Schematic protocol of the experiment. C2C12 myoblasts were induced into myotube with differentiation medium for 2 days, and then myotubes were cultured under the differentiation condition with/without TNF-α (20 ng / ml). Three days after TNF-α treatment, myotubes were stained with myosin heavy chain (MyHC) antibody (green), and myofiber diameter was measured and quantified. Represented data show that TNF-α treated myotubes were thicker than control myotubes. Values were shown as mean±SE. Scale bar = 50 μm.

(J) The protein levels of IRS1, INSR, PI3Krl, Akt in myotube, which were treated with/without TNF-α for 1 and 5 days, were evaluated by western blot analysis, revealing that protein levels of IRS1 and Akt-phosphorylation were higher in myotubes treated with TNF-α compared to control myotubes 1 day after differentiation. Five days after treatment, IRS1 level in TNF-α treated myotube had declined to the same level of that in control myotube and phospho-Akt levels were
reduced while INSR and PIK3r1 levels were not altered in TNF-α treated myotubes. All quantitative results are normalized by GAPDH, and are shown as mean±SE from four independent experiments.

(K) A model for the regulatory network involving miR-128a and IRS-Akt signaling in myogenesis. The model proposes that TNF-α regulates IRS1, PIK3r1 and phosphorylation of Akt levels through miR-128a.

Table 1  Top10 pathways that are enriched with predicted target genes of miR-128a in GO:0009987 cellular process

Supplementary figure 1 Purification of muscle satellite cells from adult mouse fore and hind limb muscles

(A) Cells were stained with CD45-FITC, CD31-PE and Vcam1-APC, and then analyzed by FACS. CD45(-) CD31(-) Vcam1(+) cells were considered muscle satellite cells. Dead cells were excluded by PI staining.

(B) CD45(-) CD31(-) Vcam1(+) cells were stained with Pax7 (red; Developmental Studies Hybridoma Bank), MyoD (green; BD Pharmingen) and DAPI (blue) before and after culture under the growth condition for 4 days in vitro. Percentages of Pax7- and MyoD-positive cells are shown. Most CD45(-) CD31(-) Vcam1(+) cells express Pax7, and express MyoD after culture in vitro. Scale bar = 50 μm.
Supplementary figure 2  Effects of miR-128a during myoblasts differentiation

Primary myoblasts where miR-128a is overexpressed or inhibited were induced to differentiate with DMEM medium containing 5% horse serum for 3 days. The differentiated cells were stained with Myosin heavy chain (MyHC) antibody (red) and DAPI (blue). Scale bar = 50 μm.

Supplementary figure 3  Inhibition of miR-128 induces hypertrophy in C2C12 myotube

C2C12 myoblasts transduced by Anti miR-128 or scrambled control lentivirus vectors were induced to differentiate for 5 days, and then stained with myosin heavy chain (MyHC) antibody (red). Scale bar = 50 μm.

Supplementary figure 4  The production of minicircle vector carrying EF1 promoter and H1 promoter with scrambled miRNA or antisense of miR-128

Minicircles are the product of site-specific recombination between the attB and attP sites driven by PhilC31 integrase. After adding 1%-L-arabinose to the bacterial culture media, the att sites undergo intramolecular recombination, resulting in producing of two circular DNAs: one is the minicircle vector, which contains H1 promoter, the gene cassettes and the right hybrid sequence (attR), and the other is the bacterial backbone, which contains the origin of replication, the antibiotic marker, and the left hybrid sequence (attL).

Supplementary figure 5  Effects of TNF-α during myoblast differentiation
(A, B) Phase contrast images (left) and myosin heavy chain staining (right) in C2C12 myotube (A) and miR-128 overexpressing C2C12 myotube (B). 

C2C12 myoblasts were induced to differentiate into myotube for 4 days with/without TNF-α (20 ng / ml). TNF-α inhibited C2C12 myoblast fusion regardless of miR-128a expression. Scale bar = 50 µm.

(C) miR-128a overexpressing C2C12 myoblasts were induced into myotube with differentiation medium for 2 days, and then myotubes were cultured under the differentiation condition with/without TNF-α (20 ng / ml). Three days after TNF-α treatment, myotubes were stained with MyHC antibody (red), and myofiber diameter was measured and quantified. Represented data show that TNF-α treatment in miR-128a overexpressing myotubes did not affect myotube fiber size relative to control myotubes. Values were shown as mean±SE. Scale bar = 50 µm.

Supplementary figure 6  Effects of insulin after TNF-α treatment in C2C12 myotubes

C2C12 myoblasts were induced to differentiate into myotubes for 5 days, and then treated with TNF-α (20 ng / ml) and/or insulin (10 µg / ml; Sigma). After treatment, proteins were extracted IRS1 expression was analyzed. These results show that treatment with insulin blocked the increase in IRS1 levels normally induced by TNF-α, suggesting that the sensitivity of IRS1 to insulin in myotubes was increased after treatment of TNF-α.

Supplementary Table 1

Primer sequences used in this study were listed.
Supplementary Table 2  GO functional enrichment of miR-128 predicted target genes

(subjected to utilized DAVID, categorized based on biological process of level 1)

Abbreviations list

miRNA, microRNA
INSR, insulin receptor
IRS1, insulin receptor substrate 1
PIK3r1, phosphatidylinositol 3-kinases regulatory 1
TNF-α, tumor necrosis factor-alpha
DMD, duchenne muscular dystrophy
UTR, untranslated region
GO, gene ontology
KEGG, kyoto encyclopedia of genes and genomes
DAVID, database for annotation, visualization and integrated discovery
DAPI, 4,6-diamidino-2-phenylindole
FACS, fluorescence activated cell sorting
GM, growth medium
DM, differentiation medium
CTX, cardiotoxin
TA, tibialis anterior
1 QC, quadriceps
2 IGFs, insulin-like growth factors
3 mTOR, mammalian target of rapamycin
4 RPS6K, ribosomal protein S6 kinase
5 GFP, green fluorescent protein
6 MyHC, myosin heavy chain
7 NF-kappaB, nuclear factor-kappaB
8 A2BAR, adenosine 2B receptor

References


Smirnova, L., Grafe, A., Seiler, A., Schumacher, S., Nitsch, R. and Wulczyn, F. G.


Figure 1

A

miR-128a expression levels (normalized by U6)

Brain
Thymus
Lung
Heart
Rectum
Testis
Spleen
Kidney
Liver
Diaphragm
Adipocyte
TA muscle

B

miR-128a expression (normalized by U6)

Q-satellite cells
Myoblast
Myotube

C

miR-128a expression (normalized by U6)

0
1
3
7
14
(Days after CTX injection)
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
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<td>miR-128a 3'</td>
<td>TTTCTCTGGCCAGTGACACT-5'</td>
</tr>
<tr>
<td>Lrs1 3'UTR(Mut)</td>
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<tr>
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B

![Graph showing relative luciferase activity with p-values]

- Control
- WT
- Mutant

i. Irs1 3'UTR
ii. Insr 3'UTR
iii. Pik3r1 3'UTR

p-values:
- 0.002
- 0.005
- 0.004

Legend:
- CON
- miR-128a
**Figure 3**

**A**

Graph showing the number of cells over different time periods. Legend includes CON, miR-128a, and Anti miR-128. Stars indicate statistical significance with *p < 0.05 (vs. CON) and # p < 0.05 (vs. miR-128a).

**B**

Images with DAPI and H3-P (+) / DAPI (%). Statistical significance indicated with p < 0.05 and p < 0.01.

**C**

Mouse myoblasts

- IRS1
- INSR
- PI3K
- Akt (Ser 473)
- Total Akt
- p21
- GAPDH

Protein expression levels for each protein with a graph showing expression for CON, miR-128a, and Anti miR-128.

**D**

Protein expression for IRS1/GAPDH, INSR/GAPDH, and PI3K/GAPDH. Graphs show expression levels for CON and miR-128a.

**E**

Primary myoblasts

Graph showing mRNA expression levels normalized by 18S rRNA for Myf5, Pax7, MyoD, and Myogenin for CON, miR-128, and Anti miR-128.
Figure 4

A. Primary myotube (differentiation day3)

B. Primary myotube (differentiation day3)

C. Primary myotube (differentiation day5)

D. C2C12 myotube (Day 1)

E. IRS1 / GAPDH

F. C2C12 myotube (Day 5)

G. C2C12 myotube (Day 5)
Figure 5

A. Graph showing body weight over days after injection. The graph compares two groups: CON and Anti miR-128. Significant differences are indicated by * (p<0.05) and ** (p<0.01) compared to CON.

B. Bar charts showing muscle weight for Quadriceps, Gastrocnemius, and Tibialis anterior. The bars are labeled for CON and Anti miR-128. Significant difference is indicated by * (p<0.05). n=4.

C. Bar chart showing mRNA expression (normalized by 18s rRNA) for IRS1, INSR, PDPK1, PIK3r1, and RPS6Kb1. The bars are labeled for CON and Anti miR-128.

D. Images showing histological cross-sections for CON and Anti miR-128. A bar graph shows percentage distribution of fiber diameters. Significant difference is indicated by * (p<0.001 vs. CON).

E. Bar chart showing mRNA expression (normalized by 18s rRNA) for Myh7, Myh2, Myh1, and Myh4. The bars are labeled for CON and Anti miR-128.
Figure 6

A

**mIR-128a expression (normalized by U6)**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>TNFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary myoblast</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
<tr>
<td>C2C12 myoblast</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

B

**H3-P (+) / DAPI (%)**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>TNFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary myoblast</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
</tr>
</tbody>
</table>

C

**Mouse myoblasts**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>+TNFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS1</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>INSR</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>PI3Kr1</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>pAkt (Ser 473)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Total Akt</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>p21</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>GAPDH</td>
<td><img src="image" alt="Image" /></td>
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</table>

D

**mIR-128a expression (normalized by U6)**

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>TNFa</th>
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<tr>
<td>C2C12 Myotube</td>
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</table>

E

**C2C12 myotube**

<table>
<thead>
<tr>
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<th>CON</th>
<th>+TNFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS1</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td>INSR</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td>PI3Kr1</td>
<td><img src="image" alt="Image" /></td>
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<td>PDK1</td>
<td><img src="image" alt="Image" /></td>
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<tr>
<td>pAkt (Ser 473)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Total Akt</td>
<td><img src="image" alt="Image" /></td>
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</tr>
<tr>
<td>GAPDH</td>
<td><img src="image" alt="Image" /></td>
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</table>

F

**Protein expression**

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<th>CON</th>
<th>TNFa</th>
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<tbody>
<tr>
<td>IRS1 / GAPDH</td>
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<tr>
<td>PI3Kr1 / GAPDH</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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<tr>
<td>pAkt / Total Akt</td>
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</table>

G

**mIR-128a expression (normalized by U6)**

<table>
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<th>TNFa</th>
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<tbody>
<tr>
<td>C2C12 myotube</td>
<td><img src="image" alt="Graph" /></td>
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</table>

H

**mIR-128a overexpressed C2C12 myotube**

<table>
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<th>CON</th>
<th>+TNFa</th>
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<tr>
<td>IRS1</td>
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<tr>
<td>PI3Kr1</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>pAkt (Ser 473)</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Total Akt</td>
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</tr>
<tr>
<td>GAPDH</td>
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Figure 6

I

<table>
<thead>
<tr>
<th></th>
<th>-1</th>
<th>0</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>GM</td>
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<td>DM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ TNFα</td>
<td></td>
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</table>

C2C12 myotube (Day 5)

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>+TNFα (Day 3)</th>
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<tbody>
<tr>
<td>Phase</td>
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<tr>
<td>Myosin heavy chain</td>
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</table>

Phases and Myosin heavy chain images for CON and +TNFα groups.

J

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 5</th>
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<tbody>
<tr>
<td>CON</td>
<td>+TNFα</td>
<td>CON</td>
</tr>
<tr>
<td>IRS1</td>
<td></td>
<td>180</td>
</tr>
<tr>
<td>INSR</td>
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<td>95</td>
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<tr>
<td>PI3Kr1</td>
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<td>85</td>
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<tr>
<td>pAkt (Ser 473)</td>
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<td>60</td>
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<tr>
<td>Total Akt</td>
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<td>60</td>
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<tr>
<td>GAPDH</td>
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<td>37</td>
</tr>
</tbody>
</table>

K

Insulin / IGF-1  

TNFα

miR-128a

PI3K

Akt

Protein synthesis

Cell proliferation
Table 1

Table 1 Top 10 pathways that are enriched with predicted target genes of miR-128a in GO:0009987 cellular process

<table>
<thead>
<tr>
<th>Term</th>
<th>Count</th>
<th>%</th>
<th>Genes</th>
<th>P-value</th>
<th>FDR</th>
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</thead>
<tbody>
<tr>
<td>Insulin signaling pathway</td>
<td>14</td>
<td>3.9</td>
<td>MKIK2, FOXO1, PDE3B, PDE3A, RPS6KB1, MAPK10, PPP1CC, IRS1, PDPK1, TSC1, GSK3B, GYS1, INSR, PIK3R1</td>
<td>3.6.E-05</td>
<td>4.1.E-02</td>
</tr>
<tr>
<td>MAPK signaling pathway</td>
<td>19</td>
<td>5.3</td>
<td>FGF14, TGFBR1, NF1, MAPKAPK1, MKIK2, MAPK10, CACNG2, ACVR1C, RPS6KA5, RPS6KA4, MAPK2, MAPK14, PDGFR1, MAPK3IP3, RAP1B, MAP3K14, MAP2K7, MAP3K12, CACNA1A</td>
<td>9.8.E-05</td>
<td>1.1.E-01</td>
</tr>
<tr>
<td>Neurotrophin signaling pathway</td>
<td>12</td>
<td>3.4</td>
<td>NTRK3, RPS6KA5, RPS6KA4, MAPK14, GSK3B, YWHAB, RAP1B, MAPK10, NGFR, MAP2K7, IRS1, PIK3R1</td>
<td>3.8.E-04</td>
<td>4.3.E-01</td>
</tr>
<tr>
<td>Pathways in cancer</td>
<td>19</td>
<td>5.3</td>
<td>DCC, E2F3, RET, HSP90AA1, FGF14, WNT3A, TGFBR1, RXRA, MET, FOXO1, MAPK10, ACVR1C, VEGFC, GSK3B, PDGFR1, RARA, RUNX1, PIK3R1, TRAF7</td>
<td>1.1.E-03</td>
<td>1.2.E+00</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>13</td>
<td>3.7</td>
<td>VAV3, MET, MAPK10, PPP1CC, VEGFC, PDPK1, ITGA5, GSK3B, PDGFR1, RAP1B, RELN, COL11A2, PIK3R1</td>
<td>3.9.E-03</td>
<td>4.3.E+00</td>
</tr>
<tr>
<td>Progesterone-mediated oocyte maturation</td>
<td>8</td>
<td>2.2</td>
<td>HSP90AA1, ADCY7, MAPK14, ADCY6, PDE3B, PDE3A, MAPK10, PIK3R1</td>
<td>5.2.E-03</td>
<td>5.7.E+00</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>8</td>
<td>2.2</td>
<td>DCC, TGFBR1, GSK3B, MET, PDGFR1, MAPK10, PIK3R1, ACVR1C</td>
<td>5.6.E-03</td>
<td>6.1.E+00</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>8</td>
<td>2.2</td>
<td>PDPK1, E2F3, HSP90AA1, CREB1, GSK3B, PDGFR1, FOXO1, PIK3R1</td>
<td>7.1.E-03</td>
<td>7.8.E+00</td>
</tr>
<tr>
<td>mTOR signaling pathway</td>
<td>6</td>
<td>1.7</td>
<td>VEGFC, PDPK1, TSC1, ULK1, RPS6KB1, PIK3R1</td>
<td>1.1.E-02</td>
<td>1.2.E+01</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>9</td>
<td>2.5</td>
<td>DCC, SEMA6A, SEMA6D, LIMK1, SEMA7A, GSK3B, MET, NFAT5, EPHB2</td>
<td>1.7.E-02</td>
<td>1.7.E+01</td>
</tr>
</tbody>
</table>