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# MiR-29c/PRKCI Regulates Axonal Growth of Dorsal Root Ganglia Neurons Under Hyperglycemia

**Longfei Jia**[1], **Lei Wang**[1], **Michael Chopp**[1,2], **Chao Li**[1], **Yi Zhang**[1], **Alexandra Szalad**[1], and **Zheng Gang Zhang**[1]

[1]Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

[2]Department of Physics Oakland University, Rochester, MI 48309, USA

## Abstract

Diabetes initially induces distal axonal damage of peripheral nerves, but molecular mechanisms that mediate axonal injury are not fully understood. MircoRNAs (miRNAs) regulate axonal growth. We found that diabetic db/db mice exhibited substantial upregulation of miR-29c in dorsal root ganglia (DRG) neurons, sciatic nerve, and foot pad tissues. Bioinformatic analysis revealed *PRKCI*, a gene that encodes a member of the protein kinase C (PKC) iota, as a putative target for miR-29c. Western blot analysis showed that diabetic mice exhibited a considerable reduction of PRKCI protein levels in sciatic nerve tissues and DRG neurons. Using dual-luciferase assay, we found that co-transfection of a plasmid containing miR-29c binding site at 3′ UTR of *PRKCI* gene and miR-29c mimics effectively reduced luminescence activity, which was abolished when miR-29c seed sequences at 3′ UTR of *PRKCI* gene were mutated. In vitro, high glucose substantially upregulated and reduced miR-29c and PRKCI protein levels, respectively, in DRG neurons, which were associated with significant reduction of axonal growth. Knockdown of endogenous miR-29c in DRG neurons by siRNAs overcame reduced PRKCI protein and axonal growth under high glucose condition. Moreover, knockdown of PRKCI in DRG neurons by siRNAs under regular glucose condition considerably inhibited axonal growth. Together, these findings suggest that miR-29c is a negative regulator of axonal growth of DRG neurons by targeting PRKCI under hyperglycemia.

### Keywords

Peripheral neuropathy; Diabetes; Axonal growth; miR-29c; PRKCI; MARCKS

## Introduction

Diabetes induces distal axonal damage of the dorsal root ganglia (DRG) neurons, which leads to the syndromes of numbness, loss of sensation, and pain at an early stage of diabetic

Correspondence to: Zheng Gang Zhang.

**Compliance with Ethical Standards**

**Conflicts of Interest** The authors declare that they have no conflict of interest.

peripheral neuropathy (DPN) [1, 2]. Neurovascular dysfunction and inflammation induced by hyperglycemia have been indicated to contribute to axonal loss [3–5]. However, the underlying mechanisms that mediate diabetes-induced distal nerve damage are not fully understood.

MicroRNAs (miRNAs) mediate gene expression through mRNA destabilization and/or translational repression, and are involved in biological function of DRG neurons and development of diabetic peripheral neuropathy [6, 7]. Conditional ablation of miRNA machinery proteins of Dicer and argonaut 2 (Ago2) in DRG neurons impairs DRG axonal growth [8–10]. Diabetes alters miRNA profiles of DGR neurons in type II diabetic mice, and high glucose also changes axonal miRNAs in cultured embryonic DRG neurons, which locally regulate axonal outgrowth [6, 8, 11]. We recently demonstrated that axonal miR-29c in cultured embryonic cortical neurons locally mediates axonal elongation [12]. In the present study, we thus investigated the role of miR-29c in regulating axonal growth of DRG neurons under hyperglycemia conditions.

Protein kinase C (PKC) is a family of protein kinase enzymes including 15 isozymes that are classified into three groups: conventional (isoforms α, βI, βII, and γ), novel (isoforms δ, ε, η, and θ), or atypical (isoforms ζ and ι/λ) [13–15]. Different isoforms of PKC play distinct roles in neural development and are involved in DPN [4]. Diabetic DRG neurons exhibit substantial reduction of PKCα activities and upregulation of PKCβII expression [16]. The isoform-selective PKCβ inhibitor, ruboxistaurin mesylate, has been shown to improve neurological outcome in animals with DPN, but clinical trials did not reach a firm conclusion due to a lack of statistical power [17]. Atypical PKCι/λ (PRCKI) is required for neural development [18–21]. However, its role in DRG axonal growth remains unclear.

Here, we reported that atypical *PRCKI* is one of the genes targeted by miR-29c, and that upregulation of miR-29c in DRG neurons by hyperglycemia suppressed axonal growth through targeting PRCKI.

## Materials and Methods

All experimental procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

### Isolation of DRG and Tissues of Sciatic Nerves and Foot Pad

DRG and tissues of sciatic nerve (SN) and foot pad (FP) were harvested from BKS. Cg-m $^{+/+}$Lepr$^{db}$/J (db/db) mice at age of 20 weeks ($n$ = 4) when these mice exhibited DPN. The db/db mouse has a point mutation in the leptin receptor gene and shows a severe depletion of the insulin-producing beta-cells of the pancreatic islets and hyperglycemia [13]. The db/db mice have been widely employed as a model to study diabetic peripheral neuropathy [13–15]. The age-matched heterozygote mice (db/m, $n$ = 4) (Jackson Laboratories, Bar Harbor, ME, USA) were used as a control.

## Culture of Primary DRG Neurons

Primarily DRG neurons were collected from 8 to 12 embryos at embryonic day 18 (ED18) of one pregnant Wistar rat ($n$ = 18 rats, Charles River Laboratories, Spencerville, OH, USA) or from 20 weeks adult mice ($n$ = 4). Cultures were prepared according to published protocols [12, 15]. Briefly, embryos were removed, and the DRG was dissected and then transferred into neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 0.05% trypsin (Thermo Fisher Scientific, Waltham, MA, USA) digestion for 30 min. DRG neurons were mechanically triturated with a Pasteur pipette for 15 times and then the cells were passed through a 70-μm cell strainer (Fisher Scientific, Hampton, NH, USA) and counted to obtain a concentration of $3\times10^7$cells/ml.

A microfluidic chamber (Xona Microfluidics, Temecula, CA, USA) was used, in which microgrooves embedded in the chamber permit only distal axons to sprout from their parental cell bodies located in the cell body compartment into the distal axonal compartment [22]. Sterilized chambers were affixed to poly-D-lysine-coated (Sigma-Aldrich, St. Louis, MO, USA) dishes (35 mm, Corning, Corning, NY). We also cultured DRG neurons in regular dishes (35 mm, Corning) to measure numbers and lengths of neurites on day in vitro (DIV) 1 and DIV2. One embryonic day 18 Wistar rat was used in each experiment, which usually includes 8–12 embryos. The DRG neurons were plated at a density of $6\times10^5$cells/ chamber or $1 \times 10^5$/dish in DMEM (Thermo Fisher Scientific) with 5% FBS (Corning). The cells were incubated for 4 h and then cultured in the medium of neurobasal (Invitrogen), 50 ng/ml nerve growth factor (NGF, Sigma-Aldrich), 2% B-27 (Invitrogen), 2 mM GlutaMax (Thermo Fisher Scientific), and 1% antibiotic-antimycotic (Thermo Fisher Scientific), 5-fluorodeoxyuridine (Abcam, Cambridge, UK) were added to the neurobasal medium to purify the neurons. For DRG neurons cultured in chamber, the medium was replaced on DIV 3 with non-5-fluorodeoxyuridine neurobasal medium. Subsequently, the growth media was changed every other day [11].

To investigate the effect of high glucose (HG) on DRG neurons, we cultured DRG neurons under HG (45 mM) condition. Neurobasal medium containing 25 mM glucose was considered as regular glucose (RG) condition. The glucose concentration at 25 mM is optimal for the growth of primary DRG neurons, which does not affect osmotic pressure [23, 24]. Both RG and HG conditions are commonly used for the in vitro hyperglycemia experiments [11, 23–25].

## Transfection of DRG Neurons

To examine the effect of miR-29c on axonal growth, cultured DRG neurons were transfected by miR-29c mimics, miR-29c hairpin inhibitors, and their corresponding controls (Dharmacon, Lafayette, CO, USA) by means of Nucleofector™ kit (Lonza, Basel, Switzerland). Briefly, transfection was performed as soon as DRG neurons were ready. MiRNA mimics, inhibitors or negative control at 200 pmol/ well were mixed with 100 μl of Nucleofector solution (Lonza). DRG neurons were added to transfection solution and then transferred into a cuvette. The program O-03 was used for electroporation [11]. Using the same electroporation protocol, DRG neurons were also transfected by siRNA against *PRKCI*, or control siRNA-A (0.1 μM, Santa Cruz, Santa Cruz, CA, USA).

### Isolation of Total RNA and Real-Time RT-PCR Analysis

Using the miRNeasy Mini kit (Qiagen, Hilden, Germany), total RNA was isolated from cultured DRG neurons (DIV6), or DRG, sciatic nerve and foot pad tissues of db/db and db/dm mice. Quantitative RT-PCR (qRT-PCR) analysis was performed on ABI 7000 and ABI ViiA 7 PCR instrument (Applied Biosystems, Foster City, CA, USA) according to published methods [25, 26]. The following Taq Man miRNA primers were used: miR-29c (mature sequence: UAGCACCAUUUGAAAUCGGUUA) and U6 snRNA (mature sequence: GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT). Briefly, for the reverse transcription, 15 μl of reverse transcription reactions were used, consisting of 1–10 ng total RNA, 5 U MultiScribe Reverse Transcriptase (Applied Biosystems), 0.5 mM each of dNTPs (Applied Biosystems), 1× reverse transcription buffer (Applied Biosystems), 4 U RNase inhibitor (Applied Biosystems), and nuclease-free water (Applied Biosystems). The running program was as follows: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min. Twenty microliter qRT-PCR reactions were used, consisting of 1xTaqMan Universal PCR Master Mix No AmpErase UNG, 1× TaqMan miRNA assay, 1.33 μl of undiluted cDNA and nuclease-free water. The running program was 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each sample was tested in triplicate, and at least three samples obtained from independent experiments were examined. Relative levels of miRNAs were calculated by means of the formula $2^{-\Delta\Delta C_T}$ after normalizing $\Delta\Delta C_T$ values to a reference miRNA U6. CT values and melt curve were checked. The method of $2^{-\Delta\Delta C_T}$ was used to calculate the relative levels [27].

### Dual-Luciferase Assay

Dual-Luciferase assay was performed according to our published protocol [12]. Briefly, a segment of the 3′ UTR of PRKCI gene encompassing the miR-29c binding site (HmiT014771, Genecopoeia, Rockville, MD, USA) was cloned into a pEZX-MT06 vector with Firefly/Renilla duo Luciferase reporter driven by a CMV promoter (Genecopoeia) (Fig. 1e). Point mutations of 3′ UTR of *PRKCI* gene in miR-29c binding site were made as TGGTGCT to GTTGGTC and confirmed by sequencing (CS-HmiT01477, Genecopoeia) (Fig. 1e). Wild type or mutant vectors were transfected into HEK293 cells by lipofectamine (Life technologies, Carlsbad, CA, USA) at a concentration of 2 mg/$10^6$ cells. To test the interaction between miR-29c and 3′ UTR of *PRKCI* gene, each vector was co-transfected by miR-29c mimics (200pM/$10^6$ cells, Dharmacon). Twenty-four hours later, the cells were lysed and treated with a Dual-luciferase assay kit (Genecopoeia). Luciferase activity was detected using a multimode microplate reader (PerkinElmer/Fusion).

### Western Blot Analysis

On DIV6, total protein samples from cultured DRG neurons were isolated according to published methods [12, 26]. Total protein samples from DRG, sciatic nerve, and foot pad tissues of db/db and db/m mice were collected using the same methods. In vitro samples from four individual microfluidic compartments were pooled for one western blot. The protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo

Fisher Scientific). Western blot was performed according to previously described methods [12, 25, 26]. Briefly, equal amounts of proteins were loaded. Primary antibodies were rabbited anti-PRKCI, (1:1000, Cell Signaling Technology, Danvers, MA, USA) and rabbit anti-Myristoylated alanine-rich C-kinase substrate (MARCKS) (1:1000, Santa Cruz). The optical density of protein bands was measured and calculated by means of Fluorchem E instrument (ProteinSimple, San Jose, CA, USA).

### Immunofluorescent Staining and Axonal Measurement

Immunofluorescent staining and measurement axons and neurites were performed as previously described [26]. Briefly, a monoclonal antibody against phosphorylated neurofilament heavy protein (pNFH) (1:500; Covance, Battle Creek, MI, USA) was used. The number and length of neurites was measured from DIV1 to DIV2. The lengths of the 15 longest axons in each chamber were measured from DIV3 to DIV5 using a microscopic computer imaging device (MCID) system. Neurons were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; 1:10,000, Thermo Fisher Scientific) for detecting cell nuclei.

### Bioinformatics and Statistical Analyses

Bioinformatics were analyzed using Ingenuity Pathways Analysis (IPA). For IPA, Fischer's exact test was used to calculate the *P* value. Other data were analyzed by using SPSS 11.5. One-way ANOVA with post hoc Bonferroni tests was used for multiple group experiment analysis. Student's *t* test was used for two group comparisons. A value of $P < 0.05$ was considered as significant. Values are presented as mean ± standard deviation (SD).

## Results

### Diabetes Upregulates miR-29c and Reduces PRKCI Protein

To examine whether diabetes affects miR-29c expression, we measured miR-29c levels in DRG, sciatic nerve, and foot pad tissues. QRT-PCR analysis showed that miR-29c levels were significantly increased in these tissues of diabetic db/db mice at age of 20 weeks compared to that of age-matched non-diabetic db/m mice (Fig. 1a).

Using the IPA and TargetScan software, we then analyzed genes putatively targeted by miR-29c. Among 3538 genes that could potentially be targeted by miR-29c, we found that 33 and 94 genes are involved in type II diabetic mellitus and axonal growth, respectively. Further bioinformatics analysis showed that only one gene, *PRKCI* encoding for PKCι/λ has been implicated in both development of type II diabetic mellitus and axonal growth (Fig. 1b). Western blot analysis revealed that PRKCI protein levels were substantially reduced in DRG, sciatic nerve and foot pad tissues of diabetic mice compared to that in non-diabetic mice (Fig. 1c–d). These data indicate an inverse relation between PRKCI proteins and miR-29c after diabetes. We thus focused on *PRKCI*, as a putative target of miR-29c. Using a dual-luciferase reporter approach, we transfected HEK293 cells with a plasmid containing miR-29c binding site at 3′ UTR of *PRKCI* gene. The luminescence analysis showed that co-transfection of the plasmid and miR-29c mimics significantly reduced luminescence activity by 68% compared to transfection by either the vector or miR-29c mimics alone. In contrast,

miR-29c mimics did not reduce luminescence activity when miR-29c seed sequences at 3′ UTR of *PRKCI* gene were mutated (Fig. 1e–f). These data indicate that miR-29c regulates expression of *PRKCI* by targeting 3′ UTR of *PRKCI* gene. Moreover, western blot showed a considerable reduction of MARCKS, a PRKCI substrate, in diabetic DRG, sciatic nerve, and foot pad tissues (Fig. 1g–h), further supporting that upregulated miR-29c by diabetes targets *PRKCI.*

## MiR-29c Suppresses Axonal Growth of DRG Neurons via PRKCI Under HG Condition

To investigate the direct effect of HG on miR-29c in DRG neurons, we performed in vitro experiments in which DRG neurons from embryonic rats were treated with HG. Quantitative RT-PCR analysis showed that HG significantly increased miR-29c levels (Fig. 2a), which was associated with substantial reduction of axonal growth of DRG neurons when they were cultured in a microfluidic device (Fig. 2b). The effect of HG on increased miR-29c was also detected in DRG neurons harvested from adult mouse (Fig. 2a). To investigate the effect of miR-29c and its target, *PRKCI* on axonal growth of DRG neurons, we cultured DRG neurons from embryonic rat under HG condition. We performed the gain- and loss-function experiments by transfecting embryonic rat DRG neurons with miR-29c mimics or inhibitors. These neurons were then cultured in a microfluidic device under HG condition and axonal length in the axonal compartment was measured daily for three consecutive days from DIV3 to DIV5. Transfection efficiency was confirmed by qRT-PCR (Fig. 2c). In addition, we found that transfection of DRG neurons with miR-29c inhibitor led to a substantial reduction of HG-increased miR-29c to a level (1.08 ± 0.12, $n = 6$ chambers/3 individual experiments/group) that was comparable to the level observed under RG condition (1 ± 0.12, $n = 6$ chambers/3 individual experiments/group). Attenuation of endogenous miR-29c by miR-29c inhibitors blocked HG-suppressed axonal growth, whereas miR-29c mimics further suppressed axonal growth compared to individual control vectors (Fig. 2d). Additionally, we examined the effect of miR-29c on neurite outgrowth of DRG neurons by culturing embryonic DRG neurons in regular dishes under HG condition. We found that transfection of DRG neurons with miR-29c inhibitors or miR-29c mimics did not significantly affect the number of neurites during DIV1–2 (Fig. 2e), but substantially increased and decreased, respectively, the lengths of neurite from DIV1 and DIV2 (Fig. 2f). Collectively, these data indicate that increased miR-29c by HG inhibits axonal growth of DRG neurons.

To examine whether PRKCI regulates axonal growth, DRG neurons were transfected by siRNA against PRKCI and then cultured under RG condition. Western blot analysis showed that siRNA against PRKCI significantly reduced PRKCI and MARCKS levels (Fig. 3a–b). Knockdown of endogenous PRKCI did not affect number of neurites (Fig. 3c) but reduced the length of neurites and axons (Fig. 3d–e), indicating that PRKCI regulates axonal growth of DRG neurons.

Next, we examined whether PRKCI is required for miR-29c-mediated axonal growth. DRG neurons were co-transfected by siRNA against PRKCI and siRNA-miR-29c or miR-29c mimics. QRT-PCR analysis showed that co-transfection of siRNA-PRKCI did not affect transfection efficiency of miR-29c inhibitors or mimics (Fig. 3f). Western blot analysis showed that knockdown of endogenous PRKCI was effective (Fig. 3g–h). Knockdown of

PRKCI significantly reduced siRNA-miR-29c enhanced neurite and axonal growth and further suppressed miR-29c mimics reduced neurite and axonal growth (Fig. 3i–j), whereas the number of neurites was not affected (Fig. 3k). These data suggest that miR-29c regulates axonal growth of DRG neurons by targeting *PRKCI*.

## Discussions

In the current study, we found that miR-29c and PRKCI levels were concomitantly significantly elevated and reduced, respectively, in DRG, sciatic nerve, and foot pad tissues of db/ db mouse. Our in vitro experiments demonstrated that miR-29c elevated by HG-suppressed axonal growth of DRG neurons via targeting *PRKCI*. These findings demonstrate for the first time that miR-29c regulates PRKCI in mediating axonal growth of DRG neurons.

Mir-29c has been shown to be involved in several diseases, including cardiac fibrosis, diabetic nephropathy, and cortical neuron development [12, 28, 29]. However, the effect of miR-29c on DRG neurons has not been extensively investigated. Our in vivo data demonstrated that diabetes upregulated miR-29c in DRG, sciatic nerve, and foot pad tissues, whereas in vitro gain- and loss-function experiments revealed that miR-29c in DRG neurons upregulated by HG blocked axonal growth. These data suggest that elevation of miR-29c in DRG neurons could impair axonal growth.

Using a set of experiments, we demonstrated that miR-29c targets *PRKCI* in DRG neurons. Bioinformatics analysis showed that miR-29c can putatively target *PRKCI*, which is involved in both type II diabetes and axonal growth. Using a dual-luciferase reporter system, we confirmed that miR-29c targets a seed region in 3′ UTR of *PRKCI* gene.

Individual isoforms of PKC play distinct roles [30]. For example, PKCα is a negative regulator for nerve function [16], whereas PKCβ contributes to nerve injuries [31]. Studies in diabetes show that levels of PKCα and PKCβII are reduced and increased, respectively, in diabetic mice [16]. In contrast to conventional PKC, studies on atypical PKC are limited. Ren et al. showed that PRKCI is critical in synaptic incorporation during long-term potentiation (LTP) [20]. Tanabe et al. demonstrated that PRKCI regulates the specification of the primary dendrites of Purkinje cells [21]. Shi et al. found that PRKCI forms a Par complex with Par3 and Par6 in the axon of neurons and regulates axonal growth [19]. Together, these studies suggest that PRKCI may have a positive effect on axonal growth cone and neuronal polarization during nervous system development. The present study showed that diabetes reduced PRKCI proteins and its substrate of MARCKS in DRG neurons. MARCKS promotes axon development [32]. Thus, our data along with others suggest that PRKCI either by itself or together with MARCKS play a role in axonal growth of DRG.

In summary, the present study demonstrates that miR-29c is a negative regulator of axonal growth of DRG neurons by targeting PRKCI under hyperglycemia, which provides new insights into molecular mechanisms underlying hyperglycemia-induced axonal damage.

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## References

1. Peltier A, Goutman SA, Callaghan BC. Painful diabetic neuropathy. BMJ. 2014; 348:g1799.doi: 10.1136/bmj.g1799 [PubMed: 24803311]
2. England JD, Gronseth GS, Franklin G, Miller RG, Asbury AK, Carter GT, Cohen JA, Fisher MA, et al. Distal symmetrical polyneuropathy: a definition for clinical research. A report of the American Academy of Neurology, the American Association of Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation. Arch Phys Med Rehabil. 2005; 86(1): 167–174. [PubMed: 15641009]
3. Cameron NE, Eaton SE, Cotter MA, Tesfaye S. Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia. 2001; 44(11):1973–1988. DOI: 10.1007/s001250100001 [PubMed: 11719828]
4. Obrosova IG. Diabetes and the peripheral nerve. Biochim Biophys Acta. 2009; 1792(10):931–940. DOI: 10.1016/j.bbadis.2008.11.005 [PubMed: 19061951]
5. Doupis J, Lyons TE, Wu S, Gnardellis C, Dinh T, Veves A. Microvascular reactivity and inflammatory cytokines in painful and painless peripheral diabetic neuropathy. J Clin Endocrinol Metab. 2009; 94(6):2157–2163. DOI: 10.1210/jc.2008-2385 [PubMed: 19276232]
6. Kantharidis P, Wang B, Carew RM, Lan HY. Diabetes complications: the microRNA perspective. Diabetes. 2011; 60(7):1832–1837. DOI: 10.2337/db11-0082 [PubMed: 21709278]
7. Natera-Naranjo O, Aschrafi A, Gioio AE, Kaplan BB. Identification and quantitative analyses of microRNAs located in the distal axons of sympathetic neurons. RNA. 2010; 16(8):1516–1529. DOI: 10.1261/rna.1833310 [PubMed: 20584895]
8. Hancock ML, Preitner N, Quan J, Flanagan JG. MicroRNA-132 is enriched in developing axons, locally regulates Rasa1 mRNA, and promotes axon extension. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2014; 34(1):66–78. DOI: 10.1523/JNEUROSCI.3371-13.2014 [PubMed: 24381269]
9. Sasaki Y, Gross C, Xing L, Goshima Y, Bassell GJ. Identification of axon-enriched MicroRNAs localized to growth cones of cortical neurons. Dev Neurobiol. 2014; 74(3):397–406. DOI: 10.1002/dneu.22113 [PubMed: 23897634]
10. Kim HH, Kim P, Phay M, Yoo S. Identification of precursor microRNAs within distal axons of sensory neuron. J Neurochem. 2015; 134(2):193–199. DOI: 10.1111/jnc.13140 [PubMed: 25919946]
11. Jia L, Wang L, Chopp M, Zhang Y, Szalad A, Zhang ZG. MicroRNA 146a locally mediates distal axonal growth of dorsal root ganglia neurons under high glucose and sildenafil conditions. Neuroscience. 2016; 329:43–53. DOI: 10.1016/j.neuroscience.2016.05.005 [PubMed: 27167084]
12. Zhang Y, Chopp M, Liu XS, Kassis H, Wang X, Li C, An G, Zhang ZG. MicroRNAs in the axon locally mediate the effects of chondroitin sulfate proteoglycans and cGMP on axonal growth. Dev Neurobiol. 2015; 75(12):1402–1419. DOI: 10.1002/dneu.22292 [PubMed: 25788427]
13. Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. Cell. 1996; 84(3):491–495. [PubMed: 8608603]
14. Okada-Iwabu M, Yamauchi T, Iwabu M, Honma T, Hamagami K, Matsuda K, Yamaguchi M, Tanabe H, et al. A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. Nature. 2013; 503(7477):493–499. DOI: 10.1038/nature12656 [PubMed: 24172895]
15. Hur EM, Yang IH, Kim DH, Byun J, Saijilafu XWL, Nicovich PR, Cheong R, Levchenko A, et al. Engineering neuronal growth cones to promote axon regeneration over inhibitory molecules. Proc Natl Acad Sci U S A. 2011; 108(12):5057–5062. DOI: 10.1073/pnas.1011258108 [PubMed: 21383151]

16. Uehara K, Yamagishi S, Otsuki S, Chin S, Yagihashi S. Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice. Diabetes. 2004; 53(12):3239–3247. [PubMed: 15561956]
17. Mochly-Rosen D, Das K, Grimes KV. Protein kinase C, an elusive therapeutic target? Nat Rev Drug Discov. 2012; 11(12):937–957. DOI: 10.1038/nrd3871 [PubMed: 23197040]
18. Shi SH, Cheng T, Jan LY, Jan YN. APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. Curr Biol. 2004; 14(22):2025–2032. DOI: 10.1016/j.cub.2004.11.009 [PubMed: 15556865]
19. Shi SH, Jan LY, Jan YN. Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. Cell. 2003; 112(1):63–75. [PubMed: 12526794]
20. Ren SQ, Yan JZ, Zhang XY, Bu YF, Pan WW, Yao W, Tian T, Lu W. PKClambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP. EMBO J. 2013; 32(10):1365–1380. DOI: 10.1038/emboj.2013.60 [PubMed: 23511975]
21. Tanabe K, Kani S, Shimizu T, Bae YK, Abe T, Hibi M. Atypical protein kinase C regulates primary dendrite specification of cerebellar Purkinje cells by localizing Golgi apparatus. J Neurosci. 2010; 30(50):16983–16992. DOI: 10.1523/JNEUROSCI.3352-10.2010 [PubMed: 21159968]
22. Taylor AM, Blurton-Jones M, Rhee SW, Cribbs DH, Cotman CW, Jeon NL. A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat Methods. 2005; 2(8):599–605. DOI: 10.1038/nmeth777 [PubMed: 16094385]
23. Russell JW, Sullivan KA, Windebank AJ, Herrmann DN, Feldman EL. Neurons undergo apoptosis in animal and cell culture models of diabetes. Neurobiol Dis. 1999; 6(5):347–363. DOI: 10.1006/nbdi.1999.0254 [PubMed: 10527803]
24. Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in Type 1 and Type 2 diabetes: many differences, few similarities. Diabetes. 2005; 54(Suppl 2):S97–107. [PubMed: 16306347]
25. Wang L, Chopp M, Szalad A, Zhang Y, Wang X, Zhang RL, Liu XS, Jia L, et al. The role of miR-146a in dorsal root ganglia neurons of experimental diabetic peripheral neuropathy. Neuroscience. 2014; 259:155–163. DOI: 10.1016/j.neuroscience.2013.11.057 [PubMed: 24316060]
26. Zhang Y, Ueno Y, Liu XS, Buller B, Wang X, Chopp M, Zhang ZG. The MicroRNA-17-92 cluster enhances axonal outgrowth in embryonic cortical neurons. J Neurosci. 2013; 33(16):6885–6894. DOI: 10.1523/JNEUROSCI.5180-12.2013 [PubMed: 23595747]
27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods. 2001; 25(4):402–408. DOI: 10.1006/meth.2001.1262 [PubMed: 11846609]
28. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A. 2008; 105(35):13027–13032. DOI: 10.1073/pnas.0805038105 [PubMed: 18723672]
29. Long J, Wang Y, Wang W, Chang BH, Danesh FR. MicroRNA-29c is a signature microRNA under high glucose conditions that targets Sprouty homolog 1, and its in vivo knockdown prevents progression of diabetic nephropathy. J Biol Chem. 2011; 286(13):11837–11848. DOI: 10.1074/jbc.M110.194969 [PubMed: 21310958]
30. Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev. 2008; 88(4):1341–1378. DOI: 10.1152/physrev.00034.2007 [PubMed: 18923184]
31. Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, Naruse K, Kasuya Y, et al. A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. Diabetes. 1999; 48(10):2090–2095. [PubMed: 10512378]
32. Xu XH, Deng CY, Liu Y, He M, Peng J, Wang T, Yuan L, Zheng ZS, et al. MARCKS regulates membrane targeting of Rab10 vesicles to promote axon development. Cell Res. 2014; 24(5):576–594. DOI: 10.1038/cr.2014.33 [PubMed: 24662485]

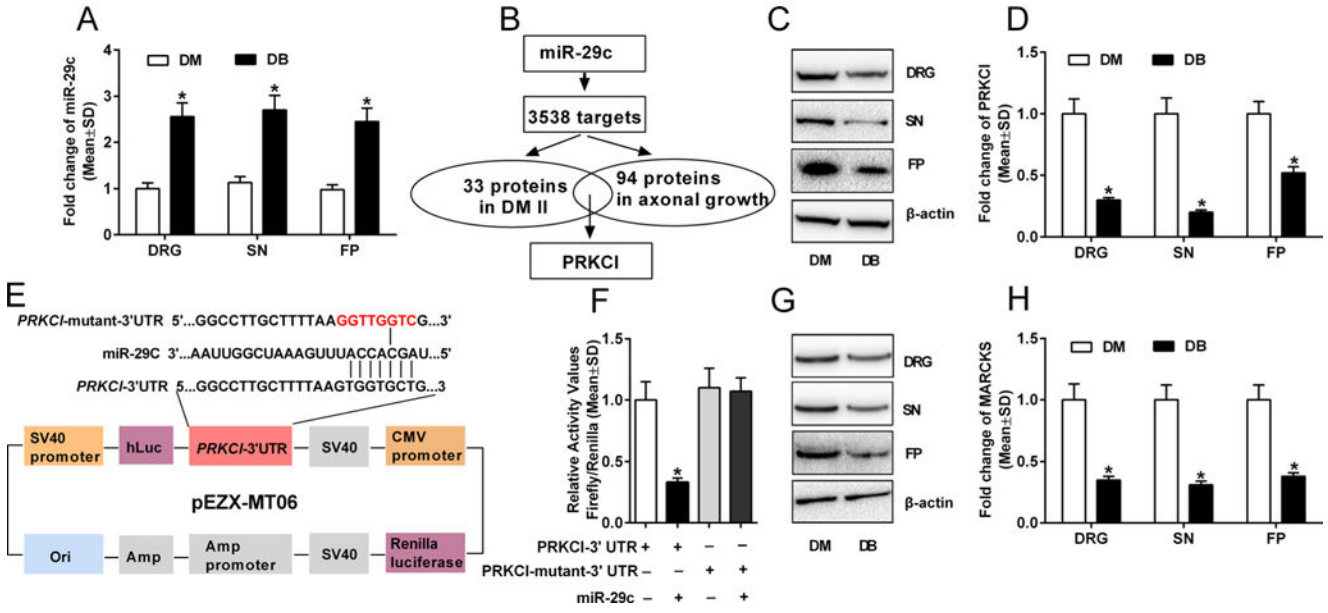


**Fig. 1.**

Upregulation of miR-29c and reduction of PRKCI protein in diabetic mice. **a** QRT-PCR analysis of miR-29c levels in DRG, sciatic nerve, and foot pad tissues of db/db or db/dm mice. **b** Schematic illustration of bioinformatics analysis of miR-29c and its putative targets. **c**–**d** Representative western blots (**c**) and quantitative data (**d**) of PRKCI in DRG, sciatic nerve, and foot pad tissues of db/db or db/dm. **e** Schematic illustration of the dual-luciferase reporter vector cloned with wild type or mutant 3′ UTR of *PRKCI*. **f** Quantitative luciferase activity data of wild type or mutant 3′ UTR of *PRKCI* with and without miR-29c mimic. **g**–**h** Representative western blots (**g**) and quantitative data (**h**) of MARCKS in DRG, sciatic nerve, and foot pad tissues of db/db or db/dm. $n = 4$ mice/group. $^*P < 0.05$. *DB* db/db, *DM* db/m, *DRG* dorsal root ganglia, *FP* foot pad, *MARCKS* myristoylated alanine-rich C-kinase substrate, *miR* microRNA, *PRKCI* atypical protein kinase C ι/λ, *SN* sciatic nerve

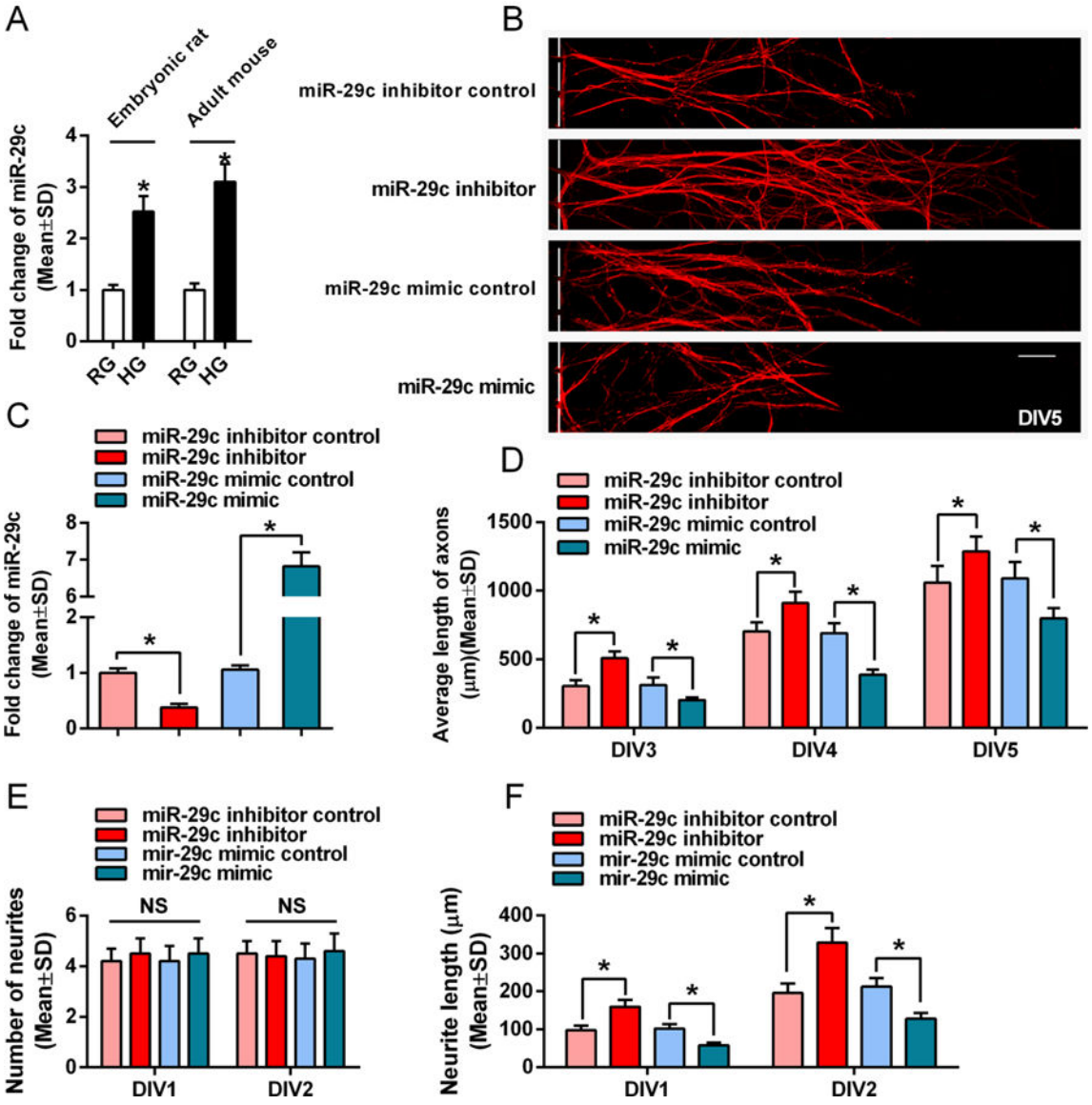


**Fig. 2.**

The effect of gain- and loss-of-function of miR-29c on axonal growth of cultured DRG neurons. **a** Quantitative RT-PCR data of miR-29c levels in DRG neurons from embryonic rat and adult mouse under regular and high glucose conditions. **b** Representative microscopic images of pNFH+ axons of embryonic DRG neurons transfected by siRNA against miR-29c (miR-29c inhibitor), miR-29c mimics (miR-29c mimic), or their corresponding controls under HG on DIV5. **c**–**d** Quantitative data of miR-29c levels (**c**) and axonal length from DIV3 to DIV5 (**d**) of embryonic rat DRG neurons transfected by siRNA against miR-29c (miR-29c inhibitor), miR-29c mimics (miR-29c mimic), or their corresponding controls under HG condition. **e**–**f** Numbers (**e**) and lengths (**f**) of neurites from DIV1 to DIV2 of embryonic rat DRG neurons transfected by siRNA against miR-29c (miR-29c inhibitor), miR-29c mimics (miR-29c mimic), or their corresponding controls under HG condition. *n* =

6 chambers/3 individual experiments/group.* $P < 0.05$. A scale bar in panel B = 100 μm. *DIV* day in vitro, *miR* microRNA, *NS* no significance, *RG* regular glucose, *HG* high glucose

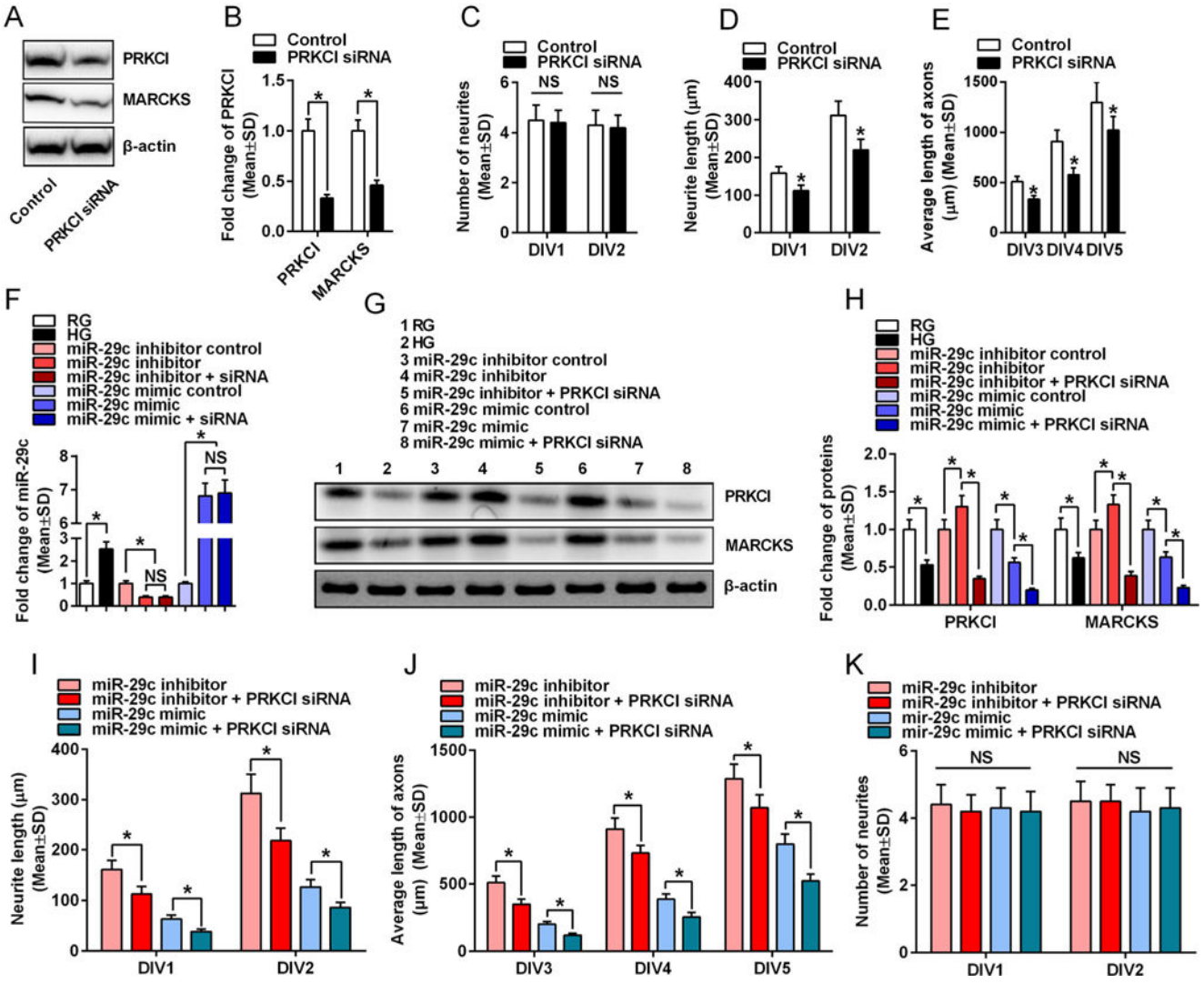


**Fig. 3.**

The effect of miR-29c and PRKCI on axonal growth of cultured DRG neurons. **a**–**b** Representative western blots (**a**) and their quantitative data (**b**) of PRKCI and MARCKS in DRG neurons transfected by siRNA against PRKCI under RG condition. **c**–**d** Numbers (**c**) and lengths (**d**) of neurite from DIV1 to DIV2 of embryonic rat DRG neurons transfected by siRNA against PRKCI under RG condition. **e** Quantitative data of axonal lengths from DIV3 to DIV5 of embryonic rat DRG neurons transfected by siRNA against PRKCI under RG condition. **f** Representative data of miR-29c levels in nontransfected DRG neurons under RG and HG, or DRG neurons transfected by siRNA against miR-29c alone (miR-29c inhibitor), siRNAs against miR-29c and PRKCI (miR-29c inhibitor + PRKCI siRNA), miR-29c mimics alone (miR-29c mimic), and miR-29c mimics and siRNA against PRKCI (miR-29c mimic + PRKCI siRNA), and their corresponding controls under RG condition. **g**–**h** Representative western blots of PRKCI and MARCKS (**g**) and their quantitative data (**h**) in nontransfected DRG neurons under RG and HG, or DRG neurons transfected by siRNA against miR-29c alone (miR-29c inhibitor), siRNAs against miR-29c and PRKCI (miR-29c inhibitor +

PRKCI siRNA), miR-29c mimics alone (miR-29c mimic), and miR-29c mimics and siRNA against PRKCI (miR-29c mimic + PRKCI siRNA), and their corresponding controls under RG condition. **i**–**k** Quantitative data of neurite lengths during DIV1 and DIV2 (**i**) and axonal lengths from DIV3 to DIV5 (**j**) or numbers of neurites during DIV1 and DIV2 (**k**) of DRG neurons transfected by siRNA against miR-29c alone (miR-29c inhibitor), siRNAs against miR-29c and PRKCI (miR-29c inhibitor + PRKCI siRNA), miR-29c mimics alone (miR-29c mimic) and miR-29c mimics and siRNA against PRKCI (miR-29c mimic + PRKCI siRNA). $n = 6$ chambers/3 individual experiments/group $^{*}P < 0.05$. *DIV* day in vitro, *HG* high glucose, *MARCKS* myristoylated alanine-rich C-kinase substrate, *miR* microRNA, *NS* no significance, *PRKCI* atypical protein kinase C ι/λ, *RG* regular glucose