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Original Paper

Nmnat 1: a Security Guard of Retinal Ganglion Cells (RGCs) in Response to High Glucose Stress

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Key Words
Diabetic retinopathy • Nmnat1 • Retinal ganglion cell • MAPK signaling

Abstract
Background/Aims: Retinal neurodegeneration is an early event in the pathological process of diabetic retinopathy (DR). Retinal ganglion cell (RGC) injury is an important pathological feature during neurodegenerative process. Protecting RGCs from high glucose-induced injury is a promising strategy for delaying or hindering diabetes mellitus-related retinal neuropathy. This study aims to investigate the role of Nmnat1, an enzyme which catalyzes a key step in the biosynthesis of nicotinamide adenine dinucleotide (NAD), in high glucose-induced RGC injury. ***Methods:*** Western blot and immunofluorescence analysis was conducted to detect Nmnat1 expression pattern in the retina and RGC-5 cell. MTT assay, Hoechst staining, trypan blue staining, and calcein-AM/ propidium iodide (PI) staining was conducted to determine the effect of Nmnat1 knockdown on RGC-5 cell function. Microarray and bioinformatics analysis was conducted to identify potential signaling pathways affected by Nmnat1 knockdown. Pharmacological intervention, molecular intervention, and *in vitro* experiments were conducted to reveal molecular mechanism of Nmnat1-mediated protective effect on RGC-5 cell function. ***Results:*** Nmnat1 is constitutively expressed in retina and RGC-5 cells. Nmnat1 knockdown aggravates RGC injury, and accelerates the development of RGC-5 cell apoptosis upon high glucose stress. MAPK signaling is the primary signaling pathway affected by Nmnat1 knockdown. Under high glucose stress, Nmnat1 knockdown leads to p38-MAPK signaling inactivation. p38-MAPK pathway inhibitor strongly blocks Nmnat1-mediated protective effect on RGC-5 cell function. ***Conclusion:*** Nmnat1 protects RGC against high glucose-induced injury via p38-MAPK signaling pathway. Nmnat1 may serve as a neuroprotective target for diabetes mellitus-related retinal neuropathy.

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Introduction

Diabetic retinopathy (DR) is the leading cause of vision impairment and blindness in both developing and developed countries [1]. Microvascular dysfunction is an important hallmark of DR. Thus, DR is often considered as a microcirculatory disease of retina [2]. Recently, increasing studies have revealed that retinal neurodegeneration is also involved in the pathogenesis of DR, which contributes to the development of microvascular abnormalities [3-5]. Understanding the mechanism of retinal neurodegeneration contributes to the development of new prevention and treatment strategies for DR.

Neural apoptosis and reactive gliosis are the important features of retinal neurodegeneration during DR [6]. Retinal ganglions cells (RGCs) located in the inner retina are the retinal neurons in which apoptotic process related to diabetes is first detected [7, 8]. RGC loss leads to a marked reduction in the thickness of retinal nerve fiber layer, which has been reported in rats with streptozotocin (STZ)-induced diabetes, and diabetic patients without or with only minimal DR [8, 9]. Thus, protecting RGCs from high glucose-induced injury could delay or hinder DR-related retinal neurodegeneration.

Nicotinamide mononucleotide adenylyltransferase (Nmnat) constitutes an NAD^+ salvage/recycling pathway using NAM as the precursor in mammalian cells. To date, three different Nmnat enzymes have been identified, including Nmnat1, Nmnat2, and Nmnat3. The function of Nmnat1 was first identified in Wallerian degeneration slow (Wld^S) mouse model. Overexpression of a chimeric nuclear protein Wld^S with Nmnat1 activity leads to axonal protective phenotype in these mice [10, 11]. Both mammalian Nmnat1 and Drosophila Nmnat1 show neuronal protective activity, although in some studies with reduced efficacy compared to Wld^S protein [12-14]. In some experimental systems, Nmnat enzymatic activity is critical for neuronal protection phenotype [12, 15]. However, the role of Nmnat1 in DR-related retinal neurodegeneration is still unclear.

In this study, we show that Nmnat1 is constitutively expressed in retina and RGC-5 cell. Nmnat1 knockdown could aggravate RGC damage, and accelerate the development of RGC apoptosis. Under high glucose stress, Nmnat1-mediated protective effect on RGC function is mainly mediated through p38-MAPK signaling pathway. Nmnat1 may become a promising therapeutic target for the treatment of DR-related retinal neurodegeneration.

Materials and Methods

Cell culture and transfection

Retinal ganglion cell line, RGC-5 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 2 mM glutamine, 10 IU/mL penicillin, 10 μg/mL streptomycin, and 10% fetal bovine serum (FBS, Gibco). They were incubated with a humidified atmosphere of 5% CO_2-95% air at 37°C. Nmnat1 siRNAs were designed and synthesized by Shanghai GenePharma Co. Ltd (Shanghai, China). Lipofectamine 2000 (Invitrogen) was used to transfect siRNAs into RGC-5 cells according to the manufacturer's instruction.

Pharmacological intervention

RGC-5 cells were transfected with Nmnat1 siRNA or Nmnat1 plasmid to change Nmnat1 expression levels. After that, RGC-5 cells were pretreated with MAPK inhibitors (U0126, 10 μM; SB203580, 10 μM; SP600125, 10 μM) for 1 h to inhibit MAPK signaling, and then exposed to high glucose (30 mM) for 48 h. U0126 (ERK inhibitor), SB203580 (p38 inhibitor), and SP600125 (JNK inhibitor) were purchased from Sigma Chemical.

Western blot analysis

RGC-5 cells were incubated with high glucose for 48 h (30 mM) with or without Nmnat1 siRNA transfection, and then washed with ice-cold PBS. These cells were homogenized in lysis buffer, and centrifuged at 12,000×*g* for 15 min. Protein concentration was detected using BCA assay. Protein (30 μg)

was separated by 8-10% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. The membranes were subsequently incubated with primary antibody and horseradish-conjugated secondary antibody. The blots were detected using the enhanced chemiluminescence (ECL) kit.

Cell viability assay

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, MTT solution (Sigma, 0.5 mg/ml) was added into each well of a 96-well plate, and incubated at 37°C for 3 h. The plates were then removed. Dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan product. The absorbance at the wavelength of 570 nm was detected using a Multi-Mode microplate reader (Molecular Device).

Hoechst staining assay

Hoechst 33258 fluorescent dye was used to detect DNA condensation and nuclear fragmentation. In brief, RGC-5 cells were (2×10^5 cells/well) were seeded onto 6-well plates for the required treatment. These cells were washed with PBS, and fixed with 4% paraformaldehyde for 15 min. They were then washed with PBS, and stained with Hoechst 33258 (mg/ml) for 15 min. Finally, these cells were observed using an Olympus IX-73 microscope.

Trypan blue staining

RGC-5 cells were seeded onto 12-well plates at the density of 5×10^4 cells/well. After the required treatment, these cells were trypsinized. 4% trypan blue solution (Gibico) was added for 10 min staining. The stained cells were observed using an Olympus IX-73 microscope.

Calcein-AM/propidium iodide (PI) staining

Calcein-AM and PI double staining was used to discriminate viable and dead cells. Briefly, RGC-5 cells were fixed with 4% paraformaldehyde for 15 min after the required treatment, and then stained with Calcein-AM solution (Molecular Probes, 10 μmol/L) for additional 15 min. After washing with PBS for three times, these cells were then stained with PI (Molecular Probes, 10 μmol/L) for 10 min. Viable cells were observed using a 490 nm excitation filter, while dead cells were observed using a 545 nm excitation filter.

Immunofluorescence analysis

Retina tissue was fixed in 4% paraformaldehyde for 1 h, immersed in 30% sucrose for 4 h, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Torrance, CA), and sectioned at 30 μm. Retinal slices were then incubated in 1% Triton X-100 in 0.1% citrate for 1 h and stained with Nmnat1 antibody conjugated with Cy-3 at 4°C overnight. Immunofluorescence labeling was then observed using an Olympus IX-73 microscope.

Microarray analysis

Total RNAs were isolated from Nmnat1-konckdown RGC-5 cells and scrambled siRNA-transfected RGC-5 cells. The cDNAs were labeled using the Superscript Plus Direct cDNA labeling system (Invitrogen), and then hybridized to the chip. Hybridization images were collected using an Agilent Microarray Scanner G2565BA. Data was first analyzed using the Agilent Feature Extraction software. Further analysis was performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Differentially expressed mRNAs were input into the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) for the annotation and functional analysis, including gene set enrichment analysis and mapping gene sets to KEGG pathway.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 software. Data was shown as mean ± SEM. Differences between different groups were analyzed by Student's *t* test or one-way ANOVA. P <0.05 was considered statistically significant.

Results

Detection of Nmnat1 expression pattern in retina and RGC-5 cell

Nmnat1 is a protein involved in mammalian NAD^+ salvage/recycling signaling. Its expression is regulated by nutrient and stress in a number of human cell lines and primary rat tissues [16, 17]. Although several studies have shown that the Nmnat1 is expressed in many tissues [18-21], it is still unknown whether it is expressed in retina and RGC-5 cell. Western blot analysis showed that Nmnat1was expressed in RGC-5 cell and in the retina of mouse and rat (Fig. 1A). Immunofluorescence experiments revealed that Nmnat1 was expressed in the inner plexiform layer, outer plexiform layer, and ganglion cell layer of mouse retina mouse (Fig. 1B). We also found that Nmnat1 was localized in both the nucleus and cytoplasm of RGC-5 cell (Fig. 1C).

Nmnat1 knockdown affects RGC-5 cell function in vitro

To reveal the functional relevance of Nmnat1 in response to high glucose stress, we determined the effect of Nmnat1 knockdown on RGC function *in vitro*. Western blot analysis showed that Nmnat1 expression was significantly reduced after Nmnat1 siRNA transfection (Fig. 2A). High glucose significantly decreased the number of viable cells (Fig. 2B) and reduced RGC-5 cell viability (Fig. 2C) as detected by trypan blue staining and MTT assay. Nmnat1 knockdown could further reduce the viability of RGC-5 cells (Fig. 2B and 2C).

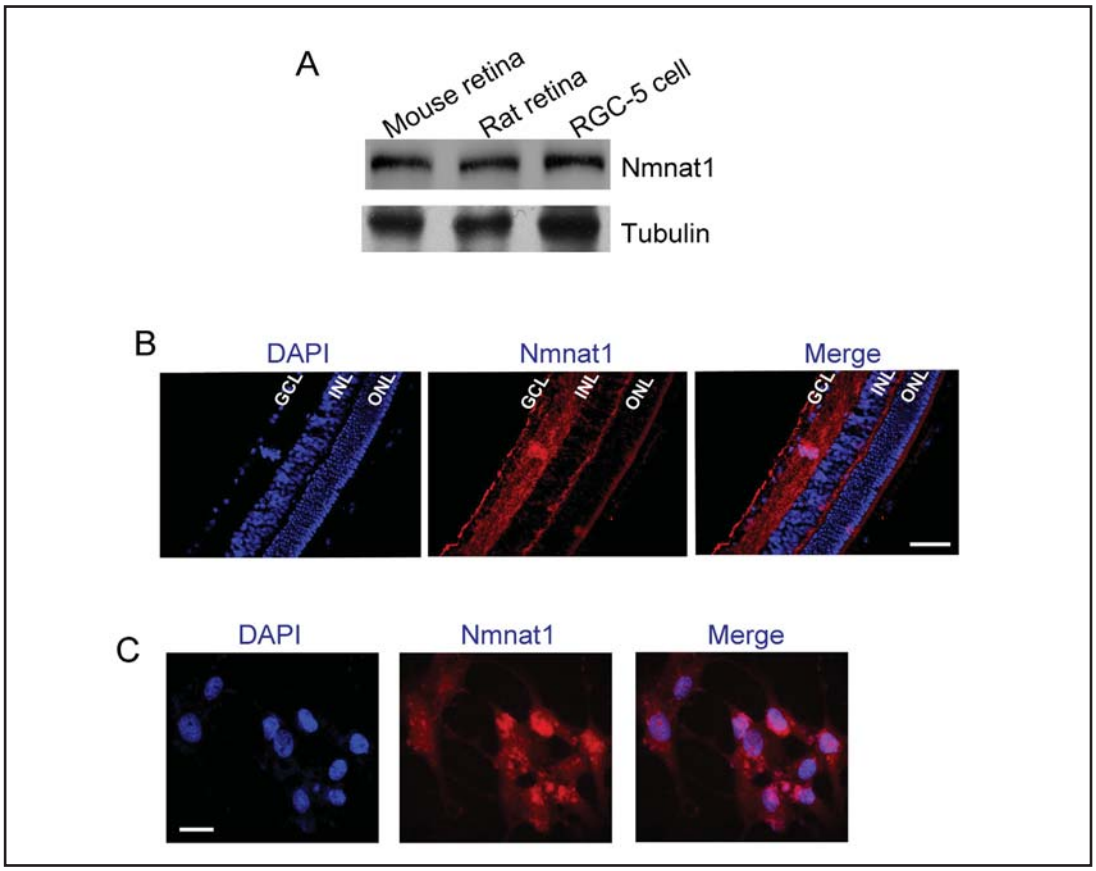

Fig. 1. Detection of Nmnat1 expression pattern in retina and RGC-5 cell. (A) Total proteins were extracted from mouse retinas, rat retinas, and RGC-5 cells. Western blots were performed to detect Nmnat1 expression. Tubulin was detected as the internal control. A representative immunoblot was shown. (B) Immunofluorescence experiments were conducted to detect Nmnat1 expression in mouse retina. Scale bar, 100 μm. (C) Immunofluorescence experiments were conducted to detect Nmnat1 expression in RGC-5 cells. Scale bar, 20 μm.

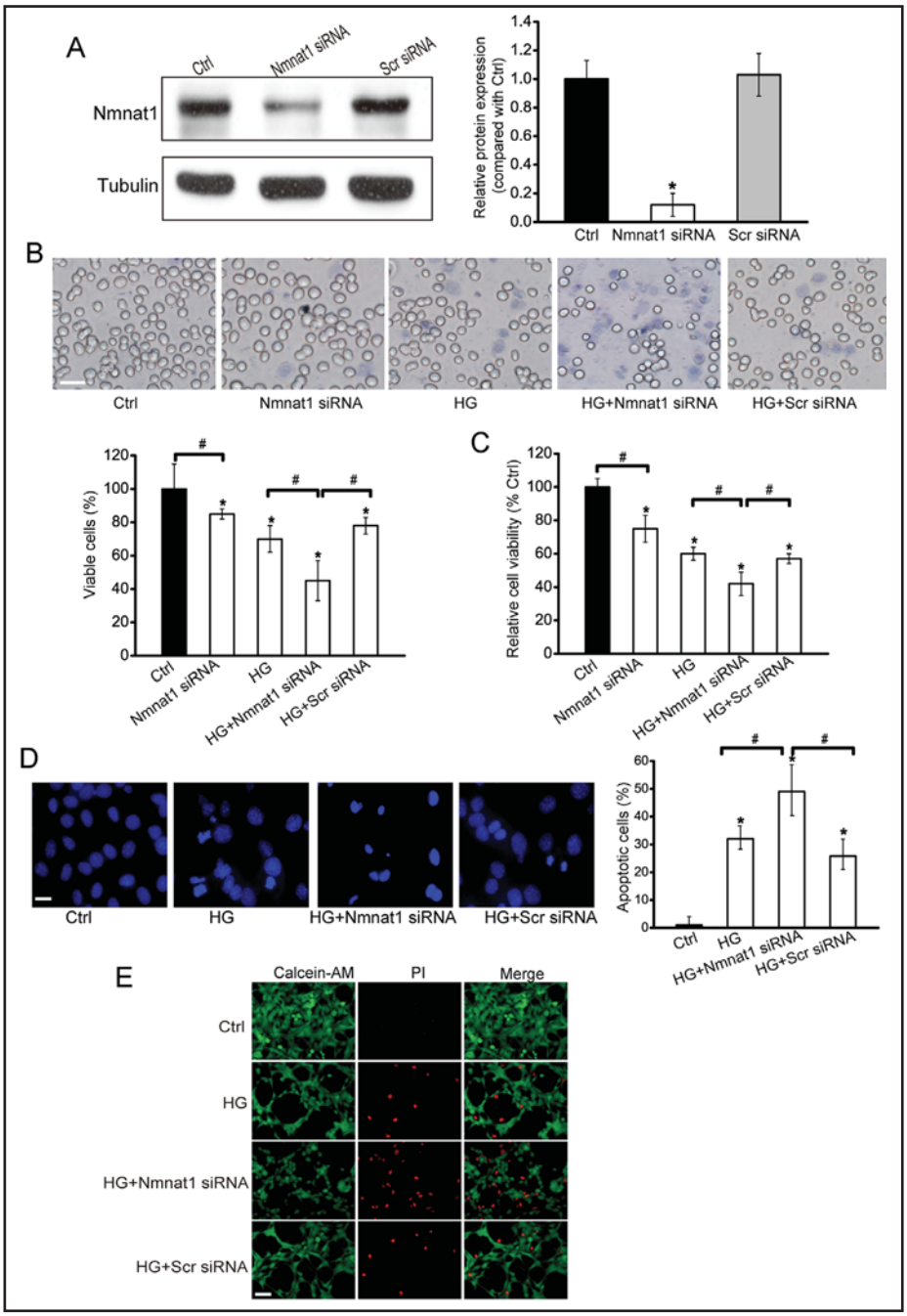

Fig. 2. Nmnat1 knockdown affects RGC-5 cell function *in vitro*. (A) RGC-5 cells were transfected with Nmnat1 siRNA, scrambled (Scr) siRNA, or left untreated (Ctrl) for 48 h. Western blot was conducted to detect Nmnat1 expression. Tubulin was detected as the interal control. A representative immunoblot was shown along with the densitometric quantitative result. (B-E) RGC-5 cells were transfected with scrambled siRNA (Scr), Nmnat1 siRNA, or left untreated (Ctrl), and then exposed with or without high glucose (30 mM) for 48 h. Viable cells were assessed by cell counting after trypan blue exclusion. Scale bar: 50 μm. (B). Cell viability was detected using MTT method (C). The data was expressed as the relative change compared with Ctrl group without high glucose treatment. (D) Apoptotic cells were analyzed using Hoechst staining and quantitated. The data was shown as means±S.E.M. and represented four independent experiments in which >300 cells were counted. Scale bar: 20 μm. (E) Apoptotic cells were analyzed using PI/calcein-AM double staining. Scale bar: 50 μm. "*" indicated a significant difference compared with the corresponding control group. "#" indicated a significant difference between the marked groups.

To determine whether Nmnat1 regulates the development of high glucose-induced apoptosis, RGC-5 cells were treated with Nmnat1 siRNA, scrambled siRNA, or left untreated, followed by high glucose treatment. Compared with high glucose-treated group, the combination of Nmnat1 knockdown and high glucose treatment resulted in higher apoptotic

Table 1. Differentially expressed genes between Nmnat1 knockdown group and wild-type group

logFC	Gene.symbol	logFC	Gene.symbol	logFC	Gene.symbol
6.217926	Cenpt	-3.5478	Ccdc91	-5.67572	Chat
5.327504	LOC679368	-3.5589	LOC100363671	-5.68078	MAP3K13
5.254431	Errfi1	-3.5675	Adra2b	-5.69721	Capn3
5.118815	Atf3	-3.5744	Olfml2a	-5.70424	Mapk4
5.036317	Ddx26b	-3.5758	Ms4a2	-5.72018	Tcf23
4.615858	Ifnb1	-3.5838	Ppl	-5.72126	Cldn16
4.59732	Fosl1	-3.5865	IgG-2a	-5.73199	Mb
4.509661	Gpr26	-3.5871	Kcnma1	-5.73413	Nppa
4.341532	Cd52	-3.624	Kcnj10	-5.74844	Hhip
4.29515	Rbfox1	-3.6279	Csn2	-5.76047	Galnt2
4.224755	LOC100912110	-3.6448	Galr1	-5.76518	Cyct
4.135874	Dusp5	-3.6625	Large	-5.77755	Kcnh1
4.103374	Nr4a3	-3.6688	Fabp6	-5.7785	Bpifb2
4.08585	Syn2	-3.6869	Adrb1	-5.7864	Prss45
4.08369	Egr4	-3.6935	Aak1	-5.79176	Cldn5
4.080494	Trim9	-3.6944	Brd1	-5.79627	LOC100365150
4.058769	Areg	-3.7008	Rgs7bp	-5.79696	Cma1
4.05522	Cct5	-3.7193	Sptssb	-5.79734	Tmcc1
4.004158	Tnfrsf17	-3.7301	Scgb1a1	-5.80592	Bmpr2
3.982108	Prkcz	-3.735	Serpinb12	-5.81269	Cpa1
3.904084	Pax4	-3.7355	Casp2	-5.81425	Rit2
3.892462	Ccrn4l	-3.7521	Serpina10	-5.83567	Chmp4b
3.886235	Atp2b1	-3.7522	Myo7b	-5.83868	Magec2
3.884896	Hes5	-3.7577	Pde4a	-5.83881	Rasgrp1
3.876654	Dsg1	-3.7577	Serpinc1	-5.83991	Galnt1
3.735942	Atp2a3	-3.7631	Mau2	-5.84145	Setd5
3.689584	Svs5	-3.7705	Eftud2	-5.84429	Dpagt1
3.679548	Spata31a5	-3.7716	Htr1a	-5.86139	Mageb16
3.465139	Cbln1	-3.7757	Chrna7	-5.87578	Rxrg
3.44877	Trpc2	-3.7806	Tln2	-5.87941	Tmem229a
3.416543	Mto1	-3.8257	Spink5	-5.88227	Gpc3
3.410994	L3hypdh	-3.8357	Ppy	-5.88709	Ntrk3
3.407081	Sit1	-3.8513	RGD1559747	-5.91016	Rhox9
3.370616	Nudt11	-3.8521	Madcam1	-5.91811	Igfl3
3.276847	Grin3a	-3.8552	Gabra4	-5.92361	Tcf20
3.235156	Chrm1	-3.8604	Nmt2	-5.92404	Fshb
3.218399	Tjp1	-3.8687	Upk3bl	-5.9266	Stard7
3.209052	Adcyap1	-3.8715	Nxf7	-5.93667	Akr1c12
3.193372	Defal1	-3.8734	Ctrl	-5.94529	Btnl8
3.175265	Cabp4	-3.8787	Agap3	-5.96222	Ass1
3.07497	Cul9	-3.8842	Hbb	-5.96848	Txnl1
2.988689	Gabrg1	-3.896	Tmco5a	-5.97198	Vill
2.982124	Ube2d4	-3.8976	Ptger4	-5.98046	Cldn1
2.959008	Calb2	-3.9029	Il10ra	-5.98278	Clic3
2.887263	Maml2	-3.9137	Btg4	-5.98495	Ildr1
2.881855	Hgfac	-3.9163	Fry	-5.98666	Nolc1
2.8658	Kctd18	-3.9187	Mylk	-5.98931	Ccdc40
2.83421	LOC683313	-3.9227	Gfra4	-5.9919	Dgkb
2.815423	Arr3	-3.9338	Fcgbpl1	-5.99286	Itih4
2.794833	Klk1c3	-3.9356	LOC102550394	-5.99972	Lama1
2.713815	Btg2	-3.9391	Cox11	-6.00347	Ppp1r1c
2.710513	Zscan20	-3.9743	Fryl	-6.01731	Mchr1
2.667522	Kif3b	-3.9754	Vom2r31	-6.02498	Pnpla7
2.656953	Ugt2b17	-3.9797	Dsg1	-6.02763	Tas2r121
2.646581	Slc34a1	-3.983	Slco2b1	-6.04209	Fut2
2.634905	Slitrk1	-3.9842	Kb15	-6.04972	Arpp21
2.629276	Ctsr	-3.9853	Smad6	-6.05215	Arhgap10
2.591693	MAP3K13	-4.0064	Kcnj5	-6.0553	Art2b
2.577252	Cmah	-4.0204	Kcnh5	-6.05613	Stard10
2.554086	Serpinb2	-4.0214	Spink5	-6.06379	Ly6g6e
2.529371	RGD1565349	-4.0228	Pou1f1	-6.07868	RGD1311595
2.513526	Tshz1	-4.0234	Prlhr	-6.09886	MAP3K1
2.48139	Fdx1l	-4.0259	RGD1565166	-6.10091	Dnah1
2.46332	Pcp2	-4.0293	Adrb3	-6.11459	Dcm5
2.462072	Ribc2	-4.0322	Sp3	-6.1168	Rab19
2.371683	Sypl2	-4.0361	Nrap	-6.12092	Shd
2.36847	Tnni3k	-4.0421	Csde1	-6.12105	Hk1
2.367445	Cyp11b1	-4.0464	Tbx2	-6.14719	Fut9
2.307372	Scyl3	-4.0552	Lrfn4	-6.16361	Vac14
2.304848	Fosb	-4.0571	Mx2	-6.16442	Pou3f3
2.297349	Nr0b2	-4.0808	RGD1306233	-6.16499	Syce3
2.1963	Pof1b	-4.0911	B3gat1	-6.1698	Mycbp2
2.1339	Kcnh7	-4.0914	Nxph4	-6.17188	Isl1
2.090766	Bcl2l2	-4.0969	P2ry4	-6.17777	LOC685527
2.07969	MAPRE1	-4.1076	Mas1	-6.18709	RGD1564664
2.056056	Megf6	-4.1224	Atp1a2	-6.19789	RGD1565959
-2.01084	Lgmn	-4.132	Lepr	-6.19964	P2rx1
-2.01157	Vom2r18	-4.1499	Gzmf	-6.22244	Slc22a24
-2.01891	Dock8	-4.1862	Fabp7	-6.23217	Sall1
-2.03276	Cd2	-4.1866	Ftsj2	-6.25936	Pth1r

logFC	Gene.symbol	logFC	Gene.symbol	logFC	Gene.symbol
-2.05312	Pvrl4	-4.2027	Cckar	-6.2667	Olr59
-2.0611	Emx1	-4.2036	Hyou1	-6.26681	Lepr
-2.09972	Klrb1b	-4.2049	Tnfsf11	-6.27064	Obp3
-2.10678	Sptb	-4.2064	Ptprf	-6.27078	LOC100125362
-2.12022	Olr414	-4.2234	Lrrc19	-6.27132	Cyp2a3
-2.12117	Slc22a8	-4.2239	Plbd1	-6.27403	Zap70
-2.15387	Bank1	-4.2305	Ceacam3	-6.2888	Fgf1
-2.15488	Znrd1as1	-4.2387	Cep104	-6.30369	Acer2
-2.16511	Slc25a39	-4.2414	Fzd5	-6.3193	Slitrk3
-2.17867	Lhfpl3	-4.2496	Fam110c	-6.3196	Akap13
-2.17901	Ctps1	-4.2533	Kcnk1	-6.32843	Tubb4a
-2.18356	Grb7	-4.2554	Snhg11	-6.34084	Obscn
-2.18579	Afmid	-4.2739	Chrna6	-6.35925	Acmsd
-2.18915	Erbb4	-4.2787	RGD1563821	-6.36611	Zdhhc22
-2.1953	Ccar1	-4.297	Eppin	-6.36618	Fam195a
-2.19584	Cnga4	-4.3045	Rere	-6.37235	Pdk4
-2.19644	Cxcr1	-4.3101	Sds	-6.3868	Tmem200c
-2.19853	Vps37b	-4.3169	Plac9	-6.38955	Inpp4b
-2.21194	Pde8b	-4.3188	RGD1311251	-6.38985	Sez6l2
-2.21462	Plin5	-4.3194	Dhrs7	-6.39283	Nat8
-2.21566	Sycn	-4.3257	Gata4	-6.39518	Igsf1
-2.22132	Grm8	-4.3291	Sult1c3	-6.39854	Nr1i2
-2.22643	Dusp13	-4.3334	Myo18a	-6.40327	Pax8
-2.23894	Cdca2	-4.3454	Rasl10b	-6.41161	LOC680590
-2.2486	MAPK6	-4.3472	Rspo3	-6.42561	Svs6
-2.25043	Ell2	-4.3547	Atp2b2	-6.43885	Gif
-2.25384	Ubqln2	-4.3606	LOC100362271	-6.44385	Hrh2
-2.29086	LOC100365462	-4.3613	Dcaf6	-6.44474	Pde1c
-2.29371	Olr1500	-4.364	Slc22a1	-6.49546	Klhl21
-2.29931	Rnf165	-4.3657	RGD1305422	-6.49826	Slc26a3
-2.3031	Fam169a	-4.3764	Fam29a	-6.50192	Arhgap28
-2.30384	Upk3a	-4.3835	Cep76	-6.50476	Pnoc
-2.32526	Gls	-4.3859	Kcng3	-6.50586	Wdr78
-2.32687	Syt7	-4.39	Adam22	-6.50742	Oprl1
-2.32811	Ptprh	-4.3936	LOC100910359	-6.51123	Sfrp4
-2.32907	Mall	-4.4196	Dnase1	-6.51678	Usp42
-2.33242	Vcsa2	-4.4212	Reg3b	-6.52285	Lrmp
-2.3401	Saa4	-4.4408	Retnla	-6.52304	Zfp870
-2.34432	Galnt2	-4.4478	Oprd1	-6.52937	Syt7
-2.34675	Masp2	-4.4616	Kcnj3	-6.53992	Tsga13
-2.3511	Kdm6a	-4.4664	Necab2	-6.55827	Grk6
-2.36142	LOC684871	-4.4676	Gpr20	-6.55899	Tbx4
-2.37277	Muc3	-4.4677	RGD1562726	-6.57034	Pon3
-2.38965	Adam6	-4.4711	Tmem182	-6.58753	Tnni3
-2.40267	Hpca	-4.48	Tubb4a	-6.58935	Tdo2
-2.40466	Dcx	-4.4909	Polr2h	-6.59458	Cars
-2.4143	Prl8a2	-4.4931	Grasp	-6.59598	LOC298139
-2.42176	Ntf3	-4.5106	Lurap1	-6.60405	Fbxo32
-2.42567	Zhx3	-4.5115	Prl8a7	-6.62567	Serpina3m
-2.43233	Ambn	-4.5116	Ms4a1	-6.62834	Fgb
-2.43876	Wfdc6a	-4.5353	Git2	-6.63235	Slc23a1
-2.43976	Dna2	-4.543	Grm2	-6.63302	Hr
-2.44023	Aplp2	-4.5516	Gcm1	-6.64113	Sec14l3
-2.45989	MAP3K5	-4.552	Krtdap	-6.64184	Atxn7l2
-2.46871	Fgf21	-4.5537	Eaf1	-6.68018	Ctnnb1
-2.47763	Usp40	-4.5639	Eci1	-6.68567	Ncan
-2.47846	Cd244	-4.5786	Apcs	-6.69843	Adora1
-2.48016	Ahsg	-4.5858	Galnt13	-6.70138	Calcb
-2.48504	Kcnd2	-4.5874	Stfa3	-6.70535	Ctsql2
-2.48504	Prl8a4	-4.5897	Smad9	-6.70593	LOC679475
-2.48614	Stac3	-4.5956	Slc8a3	-6.7151	Tpgs1
-2.48902	Htr4	-4.6034	Utrn	-6.71873	Dgcr8
-2.49134	Tnfrsf9	-4.6181	Ube3a	-6.72836	Zmym3
-2.49327	Hdgfl1	-4.6213	Acsm2a	-6.72978	Slc9a1
-2.51774	Nell1	-4.6282	Pbsn	-6.73535	LOC681005
-2.52332	Tox	-4.63	Acer2	-6.73651	Hmgcs2
-2.524	Kcnc3	-4.6332	RGD1309651	-6.75874	Hdac10
-2.5457	Car12	-4.6567	LOC290876	-6.776	Chrna10
-2.55236	Tlx2	-4.6612	Agtr2	-6.78185	Gpd1
-2.5603	Hrh3	-4.6647	Lepr	-6.78265	Tmem79
-2.569	Fhl1	-4.665	Kcne2	-6.78605	Rimbp2
-2.58007	Cd163	-4.6713	Dkk2	-6.78868	Sftpc
-2.59567	Cnr1	-4.6945	Ryr1	-6.79656	Hfe2
-2.60507	Ptprk	-4.6993	Dhrs7c	-6.81009	Sult1e1
-2.62253	Gpr135	-4.703	Apex1	-6.81041	Aplnr
-2.62301	Stpg1	-4.7162	P2ry14	-6.8108	Samd12
-2.62874	Rimbp2	-4.7261	Tcerg1l	-6.81836	Klk1c10
-2.64423	Ihh	-4.7344	Apobec2	-6.88536	L1cam
-2.67306	Tmem229a	-4.7357	Cdc45	-6.8873	Cmah
-2.67433	Sry	-4.7473	Ecel1	-6.888	RGD1309036

continued

percentage than high glucose treatment alone, as shown by increased apoptotic nuclei (condensed or fragmented) (Fig. 2D) and more PI-positive cells (dying or dead cells) (Fig. 2E).

Genome-wide analysis of differentially expressed genes between Nmnat1 knockdown cells and wild-type RGC-5 cells

To reveal the potential mechanism by which Nmnat1 regulates RGC-5 cell function, we performed microarray experiments using total RNAs from Nmnat1 siRNA-transfected RGCs-5 cells and scrambled siRNA-transfected RGC-5 cells. We found that 794 genes

logFC	Gene.symbol	logFC	Gene.symbol	logFC	Gene.symbol
-2.67582	Prdm11	-4.7555	Mto1	-6.89654	Wdr95
-2.6767	Nr4a3	-4.7595	Defa24	-6.90895	Gimap1
-2.67742	Vdr	-4.7909	LOC680406	-6.90972	Avpr2
-2.69287	Kynu	-4.7974	Glyatl2	-6.91395	Wnt10a
-2.6932	Sypl2	-4.8165	Amn1	-6.91414	Rassf10
-2.69381	Cga	-4.8265	Npffr1	-6.91451	Serpina1
-2.71283	Clcn4	-4.834	Gpihbp1	-6.92166	MAP3K10
-2.72208	Extl1	-4.8342	Incenp	-6.93953	Trhr
-2.72704	Nr2c1	-4.8384	Nkx2-5	-6.94795	Slc5a7
-2.73068	Nmnat2	-4.8407	Nubp1	-6.95339	MAPKAP1
-2.73921	LOC100911437	-4.8432	Kcnk15	-6.96479	Wfdc5
-2.74895	Fgd4	-4.8512	Dnali1	-6.99117	Ralyl
-2.75019	Sctr	-4.8535	Krt19	-7.01164	Spata19
-2.76183	Mbd4	-4.8651	Slc34a1	-7.03968	Nf2
-2.76598	Reep6	-4.8739	Notch4	-7.05073	Cpne5
-2.77191	Lrig3	-4.887	Mybphl	-7.06281	Prrt4
-2.78409	Grxcr1	-4.8897	Dip2c	-7.09496	Spata9
-2.79331	Ptger1	-4.8932	Kcnmb1	-7.11434	Crisp2
-2.79599	Gp9	-4.8988	Arhgap20	-7.11747	Cnnm1
-2.79651	LOC681335	-4.901	RGD1306750	-7.14927	Them5
-2.82588	Pde7a	-4.9018	Rbm20	-7.16217	Flvcr2
-2.8297	RGD1308195	-4.9031	Olr1078	-7.16788	Scnn1a
-2.83118	Gsg1	-4.9194	Abcc6	-7.18342	Asphd2
-2.83244	Arhgef7	-4.9328	Vstm5	-7.18546	Klra17
-2.83312	Lrfn2	-4.9363	Zfp709	-7.20407	Mroh4
-2.83486	Ccdc25	-4.9545	P4ha3	-7.24113	Myrfl
-2.83576	Grm4	-4.9557	Cabp1	-7.2926	Slco1c1
-2.84505	Gucy2e	-4.9592	Elf3	-7.294	Gnl2
-2.84817	Tgfbr3	-4.9594	Hsd3b6	-7.33032	Nrxn3
-2.85262	Nr4a2	-4.9615	Rbpms	-7.39671	Gnrhr
-2.85678	Trim66	-4.9676	Cyyr1	-7.44557	Ckmt2
-2.8617	Slc25a53	-4.9702	Cyp3a18	-7.53743	Tubb6
-2.86597	Mir144	-4.973	LOC100912109	-7.56261	Ugt2b15
-2.87157	Gpr173	-4.9737	Oprk1	-8.33459	LOC100360606
-2.87196	Cebpa	-4.9848	Lgi4	-8.4148	Letm2
-2.87812	Nrarp	-4.9885	Gab2	-8.53611	Lgals9
-2.8788	Mobp	-4.9893	Gad2	-5.67572	Chat
-2.88566	Rapgef1	-4.9928	Arhgap27	-5.68078	MAP3K13
-2.8879	Cysltr1	-4.9963	Cldn14	-5.69721	Capn3
-2.88902	Rerg	-4.9978	Pam	-5.70424	Mapk4
-2.89028	Vpreb3	-5.0218	LOC100363236	-5.72018	Tcf23
-2.8939	Hmgn5b	-5.0422	U2af2	-5.72126	Cldn16
-2.89969	Ajap1	-5.0432	Znf750	-5.73199	Mb
-2.91	Hoxc4	-5.0439	Mybpc2	-5.73413	Nppa
-2.9134	Calml5	-5.0461	Tmcc1	-5.74844	Hhip
-2.92262	Galr2	-5.0576	Ly49s7	-5.76047	Galnt2
-2.93986	Gper1	-5.0727	Sstr2	-5.76518	Cyct
-2.95224	Pde11a	-5.0895	Slc35d3	-5.77755	Kcnh1
-2.95413	LOC688090	-5.0938	Il2rb	-5.7785	Bpifb2
-2.96535	Dos	-5.0948	Clca1	-5.7864	Prss45
-2.98072	MAPKAPK3	-5.0952	Gap43	-5.79176	Cldn5
-2.98885	Olr1082	-5.1091	Pou3f1	-5.79627	LOC100365150
-2.9899	Magi3	-5.1131	Inpp5a	-5.79696	Cma1
-2.99153	Csf3r	-5.1136	Myh7	-5.79734	Tmcc1
-3.00045	RGD1309104	-5.1138	Kcnk10	-5.80592	Bmpr2
-3.0036	Sult1d1	-5.1216	Rnf207	-5.81269	Cpa1
-3.01483	Slc7a9	-5.1219	Tctex1d1	-5.81425	Rit2
-3.01552	Glod5	-5.1415	Havcr1	-5.83567	Chmp4b
-3.0222	Nefh	-5.142	Kcnc1	-5.83868	Magec2
-3.02331	Acap1	-5.1486	Trhr	-5.83881	Rasgrp1
-3.02543	Sulf1	-5.1569	Prl3d1	-5.83991	Galnt1
-3.03369	Wipf3	-5.1697	Cachd1	-5.84145	Setd5
-3.03469	Arpp21	-5.1852	Lhx5	-5.84429	Dpagt1
-3.04157	Psx1	-5.1884	RGD1307603	-5.86139	Mageb16
-3.04299	Epb41l4b	-5.1996	Cacng4	-5.87578	Rxrg
-3.04519	LOC680928	-5.209	Oacyl	-5.87941	Tmem229a
-3.04526	Ush1c	-5.2129	Klk1c7	-5.88227	Gpc3
-3.04714	Chd6	-5.2189	Prkag3	-5.88709	Ntrk3
-3.0544	Cd96	-5.2199	Mecom	-5.91016	Rhox9
-3.06132	Rap1b	-5.2241	Cnpy1	-5.91811	Igfl3

logFC	Gene.symbol	logFC	Gene.symbol	logFC	Gene.symbol
-3.06222	Ucp3	-5.2299	Nr1i3	-5.92361	Tcf20
-3.06998	Dapp1	-5.2345	Rnf125	-5.92404	Fshb
-3.07142	Bcan	-5.2368	Camkv	-5.9266	Stard7
-3.07801	Gp5	-5.2389	Vom1r101	-5.93667	Akr1c12
-3.10885	Tas2r13	-5.2561	RGD1563136	-5.94529	Btnl8
-3.11186	Syt6	-5.2565	Acan	-5.96222	Ass1
-3.1121	Cdk11b	-5.2712	Ctdspl	-5.96848	Txnl1
-3.11825	Mdga1	-5.2852	Bri3	-5.97198	Vill
-3.14509	Reg3g	-5.2882	Cpb2	-5.98046	Cldn1
-3.15143	Sftpa1	-5.2886	Ltb4r	-5.98278	Clic3
-3.15262	RGD1560784	-5.2892	Thpo	-5.98495	Ildr1
-3.15875	Fgf14	-5.3029	LOC679551	-5.98666	Nolc1
-3.16501	Pfkfb4	-5.3067	Prkag2	-5.98931	Ccdc40
-3.16759	Aldh5a1	-5.3074	RGD1310495	-5.9919	Dgkb
-3.17135	Reps1	-5.3079	Zswim8	-5.99286	Itih4
-3.17473	LOC100364488	-5.3089	Kcna2	-5.99972	Lama1
-3.18643	Irx5	-5.3163	Adipoq	-6.00347	Ppp1r1c
-3.19983	Ripply1	-5.3209	Asic4	-6.01731	Mchr1
-3.20094	Ccr4	-5.3231	MAPK7	-6.02498	Pnpla7
-3.22925	MAP3K12	-5.3373	Dnajc2	-6.02763	Tas2r121
-3.23557	Hoxa1	-5.3403	RGD1564614	-6.04209	Fut2
-3.24943	RGD1624210	-5.3419	Cel	-6.04972	Arpp21
-3.2642	Adra2c	-5.3497	Cd8b	-6.05215	Arhgap10
-3.26422	Atp1b4	-5.3499	Zg16	-6.0553	Art2b
-3.28484	Pnp	-5.356	Tal1	-6.05613	Stard10
-3.28695	Ucn2	-5.357	Drp2	-6.06379	Ly6g6e
-3.28786	Tceal6	-5.3667	LOC685577	-6.07868	RGD1311595
-3.29042	Fgd5	-5.3725	LOC246267	-6.09886	MAP3K1
-3.30854	Olr857	-5.38	Ptf1a	-6.10091	Dnah1
-3.31677	Olr287	-5.3831	Prkcg	-6.11459	Dcm5
-3.32567	Neurl1b	-5.3918	Sin3a	-6.1168	Rab19
-3.33347	Ppm1l	-5.3933	LOC100365339	-6.12092	Shd
-3.33557	Spsb3	-5.3936	Smyd1	-6.12105	Hk1
-3.33772	Fam167b	-5.4213	Gabra5	-6.14719	Fut9
-3.34391	Isl1	-5.4274	Aunip	-6.16361	Vac14
-3.3481	Mep1a	-5.4281	MAP3K3	-6.16442	Pou3f3
-3.34958	Dnali1	-5.4323	RGD1565959	-6.16499	Syce3
-3.3609	Dmrt2	-5.4399	MAP2K1	-6.1698	Mycbp2
-3.36777	S100a8	-5.4675	Itgb5	-6.17188	Isl1
-3.37193	MAP3K5	-5.4727	En2	-6.17777	LOC685527
-3.3742	Tnnt2	-5.4751	Nkx2-2	-6.18709	RGD1564664
-3.38842	Crb3	-5.4878	Mro	-6.19789	RGD1565959
-3.38854	Ppp3ca	-5.5056	Igf2	-6.19964	P2rx1
-3.39656	Zfp90	-5.5164	Dlx5	-6.22244	Slc22a24
-3.40028	Ryr2	-5.5195	Tmem179	-6.23217	Sall1
-3.40872	Tas2r118	-5.5237	LOC680988	-6.25936	Pth1r
-3.42454	Il2ra	-5.5246	Padi4	-6.2667	Olr59
-3.42901	Htr7	-5.5373	Hsd17b6	-6.26681	Lepr
-3.43339	Gad1	-5.538	Gpr158	-6.27064	Obp3
-3.43673	Pcbp3	-5.5454	Olr442	-6.27078	LOC100125362
-3.45162	Rnase2	-5.5456	Cldn6	-6.27132	Cyp2a3
-3.45567	Sv2b	-5.5504	LOC686124	-6.27403	Zap70
-3.45677	Unc13c	-5.5518	Cdk2ap1	-6.2888	Fgf1
-3.45805	Drd5	-5.5613	Ngdn	-6.30369	Acer2
-3.47398	Kmo	-5.5688	Prss42	-6.3193	Slitrk3
-3.47667	Micu2	-5.5699	RGD1560559	-6.3196	Akap13
-3.47957	Ky	-5.5742	Cldn1	-6.32843	Tubb4a
-3.49577	Trim63	-5.5854	Best2	-6.34084	Obscn
-3.49717	Slc30a2	-5.5904	Ccdc117	-6.35925	Acmsd
-3.50048	Asic1	-5.5986	Rem2	-6.36611	Zdhhc22
-3.5037	Trim9	-5.6037	Ahr	-6.36618	Fam195a
-3.50555	Greb1	-5.6038	Cacng2	-6.37235	Pdk4
-3.50587	Scn4a	-5.608	Dmrtc1c1	-6.3868	Tmem200c
-3.50762	Gkn2	-5.6104	Fam155a	-6.38955	Inpp4b
-3.51757	Pfkfb2	-5.625	RGD1310209	-6.38985	Sez6l2
-3.52576	Cxcr5	-5.6483	Dpysl4	-6.39283	Nat8
-3.52672	Oaz3	-5.6595	Cldn22	-6.39518	Igsf1
-3.52782	Gnaz	-5.6658	Fam181b	-6.39854	Nr1i2
-3.53681	Eri2	-5.667	Muc13	-6.40327	Pax8
		-3.5478	Ccdc91	-6.41161	LOC680590

were differentially expressed between Nmnat1 knockdown group and scrambled siRNA-transfected group, including 641 down-regulated genes and 76 up-regulated genes (Table 1). We next conducted GO analysis, which covers the following 3 domains, including cellular component, biological process, and molecular function. The highest enriched GO terms targeted by differentially expressed transcripts included the regulation of transcription (biological process), transcription regulator activity (molecular function), and plasma membrane (cellular component) (Fig. 3). KEGG pathway analysis showed that MAPK signaling was the top pathway enriched among these differentially expressed mRNAs (Table 2). These results suggest that MAPK signaling pathway may harbor significance and/or contribute to the pathogenesis of Nmnat1 knockdown-mediated RGC dysfunction.

Nmnat1 protects RGC-5 cells against high glucose-induced injury through p38-MAPK signaling

The above-mentioned results suggest that MAPK signaling pathway seems to emerge as a major signaling pathway involved in regulating RGC-5 cell function. We then conducted western blots to verify MAPK signaling activation *in vitro*. We showed that high glucose treatment resulted in an obvious increase in phosphorylated p38 and phosphorylated

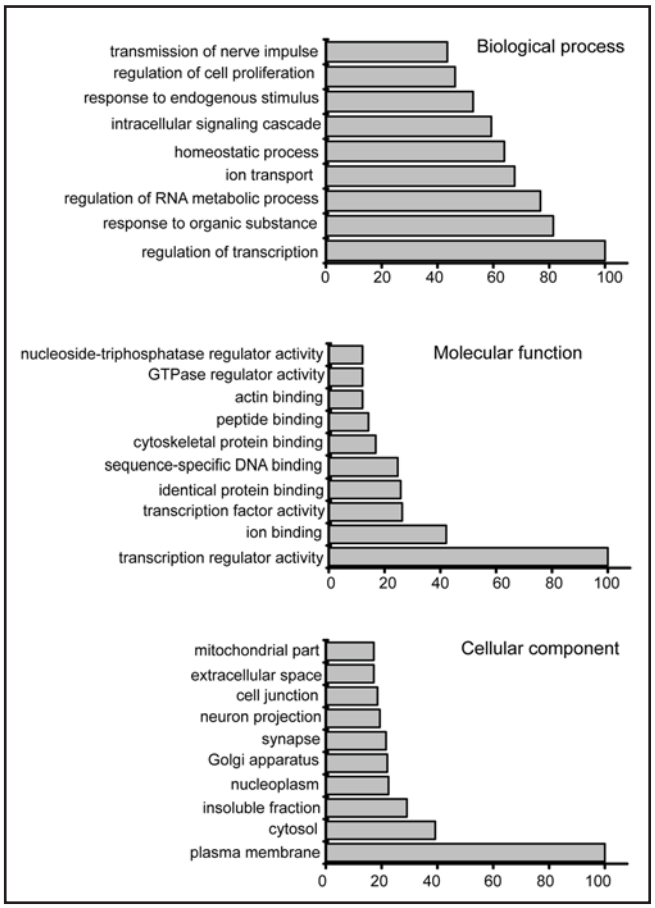

Fig. 3. Genome-wide analysis of differentially expressed genes between Nmnat1 knockdown cells and wild-type RGC-5 cells. Microarray analysis was conducted to compare gene expression difference between Nmnat1 siRNA-transfected RGC-5 cells and scrambled siRNA-transfected RGC-5 cells. Gene enrichment (GO) analysis was used to reveal the biological modules of differentially expressed mRNAs, including biological process, cellular component, and molecular function.

Table 2. Top 10 signaling pathways affected by Nmnat1 knockdown

Term	P Value	Fold Enrichment
MAPK signaling pathway	3.48E-13	3.137629097
Neuroactive ligand-receptor interaction	1.15E-06	2.710084034
Calcium signaling pathway	0.002031	2.276470588
Tight junction	0.028798	1.814198783
Wnt signaling pathway	0.029715	4.110294118
Leukocyte transendothelial migration	0.044901	1.858567775
Endocytosis	0.060841	1.554141059
Cell adhesion molecules (CAMs)	0.063781	1.666335453
rno04930:Type II diabetes mellitus	0.07389	2.348739496
rno04060:Cytokine-cytokine receptor interaction	0.087847	1.502239474

ERK1/2 levels. Nmnat1 knockdown significantly reduced phosphorylated p38 levels, but had no effect on the levels of phosphorylated ERK1/2 or JNK1/2 (Fig. 4A and 4B), suggesting that Nmnat1 knockdown leads to inaction of p38-MAPK signaling inaction. To further examine whether p38 is involved in Nmnat1-mediated protective effect on RGC-5 cell function, Nmnat1 was overexpressed in RGC-5 cells. These cells were pretreated with SB203580, U0126, or SP600125, and then exposed to high glucose for 48 h. MTT assays showed that SB203580, a p38-MAPK signaling inhibitor, strongly blocked Nmnat1-mediated protective effect on RGC-5 cell function, whereas U0126 (ERK inhibitor) or SP600125 (JNK inhibitor) treatment did not affect Nmnat1-mediated protective effect on RGC-5 cell function (Fig. 4C). Hoechst 33342 staining showed that Nmnat1 overexpression decreased high glucose-

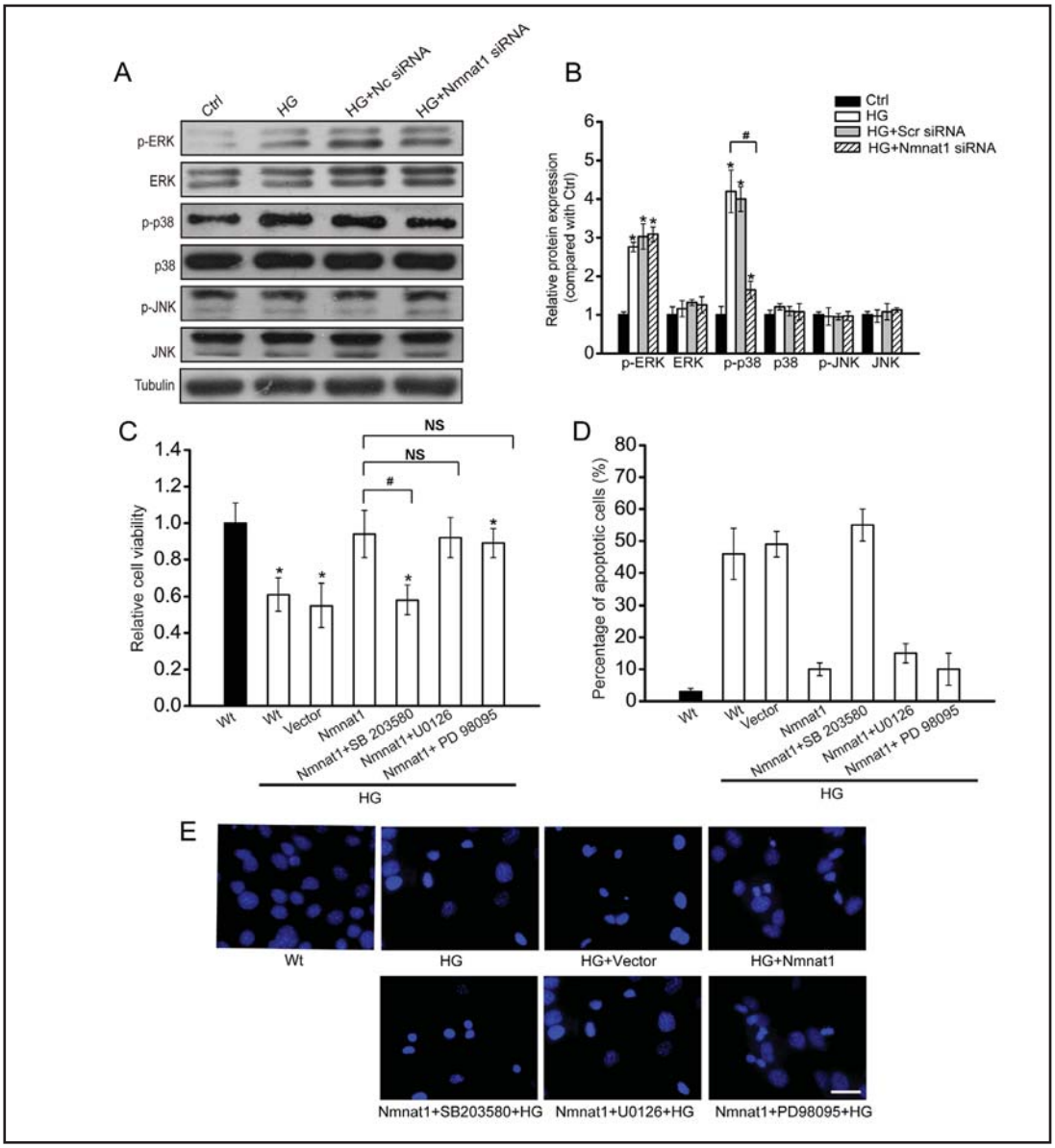

Fig. 4. Nmnat1 protect RGC-5 cells against high glucose-induced injury through p38-MAPK signaling. (A, B) RGC-5 cells were transfected with Nmnat1 siRNA, scrambled siRNA (Scr), or left untreated, and then exposed to high glucose (30 mM) for 48 h. The untreated group was taken as the control group (Ctrl). Representative immunoblots (A) of total p38, total ERK, total JNK, p-p38, p-ERK, p-JNK, and tubulin were shown along with the densitometric quantitative results (B). (C) RGC-5 cells were transfected with Nmnat1 plasmid or vector (pcDNA3.0), then pretreated with SB203580, U0126, or SP600125 for 1 h, and finally exposed to high glucose (30 mM) for additional 48 h. Cell viability was detected by MTT assay (n = 4). (D, E) RGC-5 cells were treated as shown in Fig. 4C. Apoptotic cells were analyzed using Hoechst staining and quantitated. The data was shown as means ± S.E.M. and represented four independent experiments in which > 300 cells were counted. Statistical result was shown in Fig. 4D. A representative image for Hoechst staining was shown Fig. 4E. Scale bar: 20 μm. *indicated a significant difference compared with wild-type (WT) group. #indicated a significant difference between the marked groups. NS: no significant difference.

induced RGC-5 cell apoptosis. SB203580 treatment could interrupt this protective effect, whereas U0126 or SP600125 treatment had no effect on this effect (Fig. 4D). Collectively, these results suggest that p38-MAPK signaling is involved in Nmnat1-mediated protective effect on RGC-5 cell function.

Discussion

Nmnat1 encodes an enzyme which catalyzes a key step in the biosynthesis of nicotinamide adenine dinucleotide (NAD), which is highly conserved from archaebacteria to human [22]. Studies in Drosophila and mice have revealed a neuroprotective role of Nmnat1 in neurodegenerative conditions including tauopathies, Charcot-Marie-Tooth (CMT) disease, Parkinson's disease, and glaucoma. Loss of Nmnat1 could lead to rapid and severe neurodegeneration [23-26]. In this study, we reveal a role of Nmnat1 in diabetes mellitus-related retinal neurodegeneration. Nmnat1 could protect RGCs against high glucose-induced injury *in vitro*.

DR is not only a microvascular disease but also a neurodegenerative disease [27]. Hyperglycemia could lead to the apoptosis of both retinal neurons and vascular cells [28, 29]. RGCs are located in the inner retina, and are easily injured in response to high glucose stress. Nmnat1 knockdown could aggravate RGC injury, and accelerate the development of RGC apoptosis, suggesting a protective role of Nmnat1. A recent study has revealed that cytoplasmic overexpression of Nmnat1 could protect mouse retinal ganglion cell axons and soma against from glaucomatous and ischemic-induced injury [30], also suggesting a protective role of Nmnat1 under stress condition. Taken together, Nmnat1 is emerged as a security guard of RGCs in response to stress.

To understand the mechanism of Nmnat1-mediated protective effect on RGC function, we conducted genome-wide analysis of gene expression through microarray, which can simultaneously detect the expression of thousands of transcripts. We found that the highest enriched GOs targeted by differentially expressed mRNAs were regulation of transcription (ontology: biological process), plasma membrane (ontology: cellular component), and transcription regulator activity (ontology: molecular function), suggesting that Nmnat1 knockdown may affect gene transcription, which in turn affects the expression of genes associated with RGC function. Signaling pathway analysis provides the knowledge about genomes and the relationship to biological system. We show that these differentially expressed mRNAs are most enriched in MAPK signaling pathway. MAPK family is a common participant in mediating growth factor receptor as well as adhesive and tensional signals from the cell surface to the nucleus. They are activated in response to physiological angiogenic stimuli, such as elevated shear stress and mechanical stretch [31, 32]. The activation of MAPK signaling in RGCs may be responsible for diverse downstream actions such as cell proliferation, cell apoptosis, and electrical signal transduction.

Microarray and bioinformatics analysis suggests that Nmnat1 knockdown affects the activity of MAPK signaling. Thus, we investigated the involvement of MAPK signaling in Nmnat1-mediated protective effect on RGC function. MAPKs are a family of serine/threonine kinases, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK [31, 33]. Nmnat1 knockdown leads to the inaction of p38 MAPK signaling. SB203580, a p38 MAPK pathway inhibitor, strongly blocks the effect of Nmnat1-mediated protective effect on RGC function, suggesting that p38-MAPK signaling is mainly involved in Nmnat1-mediated protective effect in RGC function. MAPK signaling activation has been reported to contribute to neuroprotection, and be involved in the development of diabetic retinopathy [34-37]. MAPK signaling could regulate a variety of cellular activities, including proliferation, differentiation, survival, and death. These biological processes may co-regulate to cope with diverse extracellular and intracellular stimuli [31]. In this study, we show novel evidence that MAPK signaling is involved in neuroprotection.

In conclusion, we show that Nmnat1 knockdown could aggravate RGC injury, and accelerate the development of RGC apoptosis in response to high glucose stress. Nmnat1 protects RGC against high glucose-induced injury via p38-MAPK signaling pathway. Nmnat1 may serve as a neuroprotective target for diabetes mellitus-related retinal neuropathy.

Acknwoledgments

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Disclosure Statement

None declared.

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In the article by Zhou et al., entitled "Nmnat 1: a Security Guard of Retinal Ganglion Cells (RGCs) in Response to High Glucose Stress" [Cell Physiol Biochem 2016;38:2207-2218 (DOI: 10.1159/000445576)] there is an error in Figure 2. The correct figure and the legend is reproduced correctly here. The authors sincerely regret this error due to wrong submission during the review process.

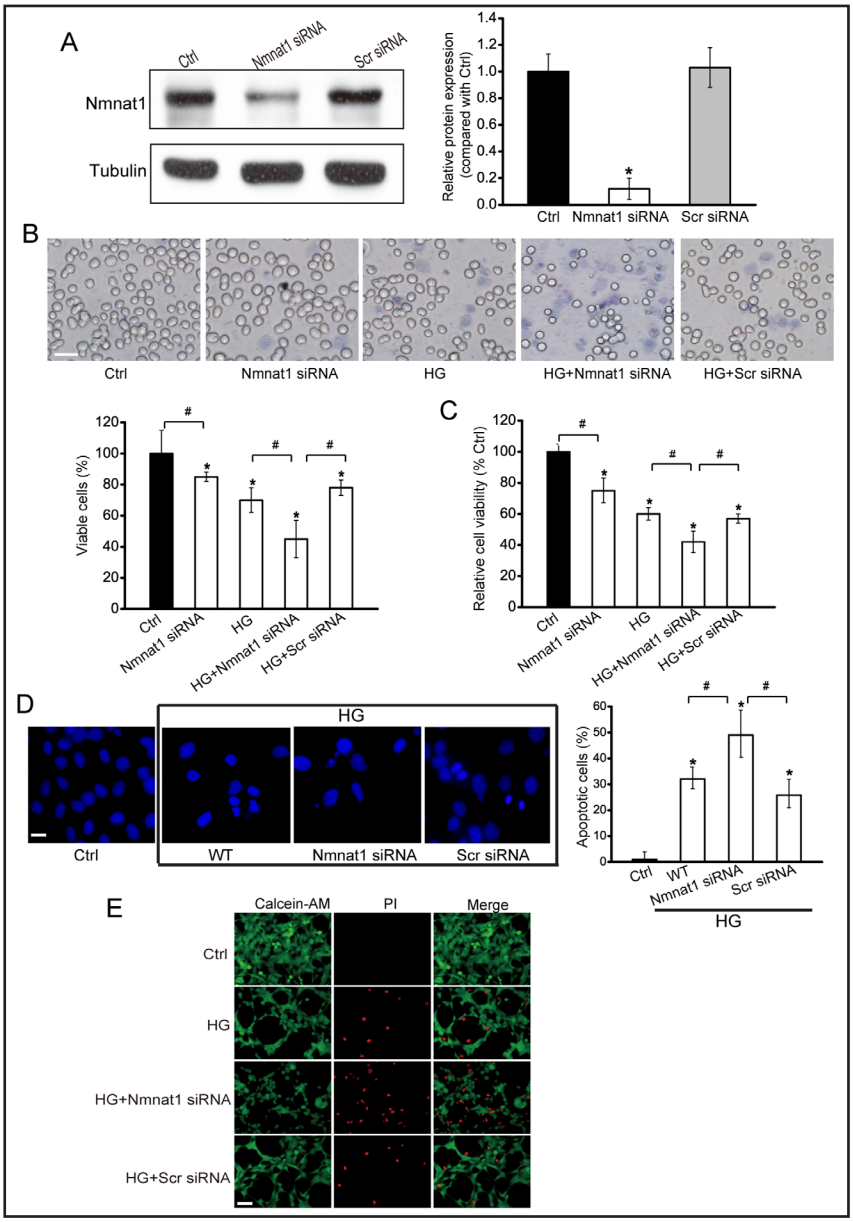

Fig. 2. Nmnat1 knockdown affects RGC function *in vitro.* (A) RGCs were transfected with Nmnat1 siRNA, scrambled (Scr) siRNA, or left untreated (Ctrl) for 48 h. Western blots were performed to detect Nmnat1 expression. Tubulin was detected as the internal control. A representative immunoblot was shown along with quantitative result. (B-E) RGCs were transfected with Scr siRNA, Nmnat1 siRNA, or left untreated (Ctrl), and then exposed with or without high glucose (30 mM) for 48 h. Viable cells were detected by cell counting after trypan blue exclusion. Scale bar: 50 μm. (B). Cell viability was detected using MTT method (C). The data was expressed as relative change compared with Ctrl group without high glucose treatment. (D) Apoptotic cells were analyzed using Hoechst staining and quantitated. The data was shown as means±S.E.M. and represented four independent experiments in which >300 cells were counted. (E) Apoptotic cells were analyzed using PI/Calcein-AM double staining. Scale bar: 50 μm. "*" indicated significant difference compared with the corresponding control group. "#" indicated significant difference between the marked groups.