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Original Research

RNA-binding protein hnRNPR reduces neuronal cholesterol levels by binding to and suppressing HMGCR

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Recent studies have identified multiple RNA-binding proteins tightly associated with lipid and neuronal cholesterol metabolism and cardiovascular disorders. However, the role of heterogeneous nuclear ribonucleoprotein R (hnRNPR) in cholesterol metabolism and homeostasis, whether it has a role in regulating 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), is largely unknown. This research identifies hnRNPR as a repressor of HMGCR. Knockdown and overexpression of hnRNPR in cultured neuroblastoma cell (N2a) and MN1 cell lines enhances and inhibits HMGCR *in vitro*, respectively. hnRNPR may exert its repressive activity on HMGCR mRNA and protein levels by using its RNA recognition motif (RRM) in recognizing and modulating the stability of HMGCR transcript. Our RNA immunoprecipitation and luciferase reporter assays demonstrate a direct interaction between hnRNPR and HMGCR mRNA. We also demonstrated that hnRNR binds to the 3' untranslated region (3' UTR) of HMGCR and reduces its translation, while hnRNPR silencing increases HMGCR expression and cholesterol levels in MN1 and N2a cells. Overexpression of HMGCR significantly restores the decreased cholesterol levels in hnRNPR administered cells. Taken together, we identify hnRNPR as a novel post-transcriptional regulator of HMGCR expression in neuronal cholesterol homeostasis.

Keywords

Heterogeneous nuclear ribonucleoprotein; 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Neuronal cholesterol biosynthesis; Neuroblastoma cell; MN1 cell; RNA metabolism

1. Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of RNA-binding proteins that bind to newly formed transcripts with crucial roles in all aspects of (pre)mRNA processing, including transcription, packaging, splicing, stabilization, subcellular transport, translational control, and degradation [1]. The role of hnRNPs in regulating gene expression has gained profound interest in disease research in the past few decades. The expression level of hnRNPs is altered in many types of neoplasm, suggesting their role in tumorigenesis [2]. In addition to cancer, many hnRNPs were also linked to various neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and frontotemporal lobar dementia (FTLD) [3–5]. Physiologically, hnRNPR regulates immunity factors [6, 7], transcription, and degradation of c-fos mRNA [8, 9]. Pathogenic variants can precipitate neurodegeneration through a mechanism involving excessive stress granule formation, or developmental defects, through unknown mechanisms [10]. A better understanding of the cellular and molecular mechanisms underpinning brain diseases is essential for drug design, development, and treatment of neurodegenerative diseases. Their associated comorbidities and considerable evidence have demonstrated the neuronal functions of hnRNPR. hnRNPR plays a crucial role in neuronal development with the highest expression at the embryonic stage, diminishing postnatally [11–13]. The functional consequence of cellular and animal physiological processes of hnRNPR perturbation are fatal, and these fatalities are not unconnected with the dysfunction of its essential physiological functions. hnRNPR overexpression encourages neurite outgrowth in PC12 cells and is also needed to bind SMN to β-actin mRNA. hnRNPR mediated axonal b-actin mRNA translocation plays an essential physiological role for axon growth and presynaptic differentiation. hnRNPR colocalizes with SMN in axons and in presynaptic terminals both *in vivo* and *in vitro*. It facilitates the transport of SMN proteins, an important protein for motor neuron survival. The deficiency of this essential protein causes Spinal muscular atrophy (SMA), the predominant form of motor neuron disease in children and young adults. hnRNPR is a potentially important regulator of neuronal homeostasis. Their disrup-

tion could impair distinct pathways in the central nervous system axis, thus confirming the importance of their conservation during evolution. In the animal model of zebrafish, knockdown of hnRNPR leads to severe disturbance of axonal elongation and its pathways which causes axonal defects in motor neurons, as demonstrated by Glinka and co. Again, phenotypically, individuals with truncating or missense variants in the same C-terminal region of hnRNPR present with multisystem developmental defects including abnormalities of the brain and skeleton, dysmorphic facies, seizures as reported by [10].

Despite the aforementioned plethora of growing literature associated with the neuronal expression profiles and functions of hnRNPR and its related neurodegenerative disorders as well as enormous studies churned out on the roles of RBPs in lipid and neuronal cholesterol metabolism as exquisitely demonstrated by many researchers in the field [14–20], the role of hnRNPR in lipid metabolism especially cholesterol is yet to be elucidated. Therefore, we take advantage of this research lacuna to hypothesize that hnRNPR may have a role to play in cholesterol metabolism. We tested this hypothesis by performing an amplex red cholesterol assay. Our assay demonstrated that hnRNPR could modulate neuronal cholesterol by up and downregulation. We next sought to determine the binding affinity and binding motifs of hnRNPR with cholesterol biosynthetic genes. Therefore, we screened all enzymes involved in the cholesterol biosynthesis pathway with the binding motif of hnRNPR by checking these genes' mRNA and protein levels in hnRNPR overexpression and knockdown samples. The rationale for doing this is to enable us to ascertain the actual genes that have binding motifs of hnRNPR as this will help us leverage the next set of genes or experiments to focus on. Using the Ensembl genome browser, the following cholesterol biosynthetic genes were identified: CYP51A1, FDFT1, HMGCR, and NSDHL, and were subsequently screened by checking their expression levels. Out of the screened genes, only HMGCR shows a significant upregulation and downregulation of mRNA and protein levels when hnRNPR is knockdown or overexpression, respectively. Another reason for HMGCR for further work is that HMGCR is the rate-limiting, irreversible, and classical committed step enzyme in cholesterol biosynthesis. It is also the most tightly regulated part controlling entry into the cholesterol biosynthetic pathway.

Compared to HMGCR, some of the other enzymes screened here are noncommittal and promiscuously branch off, resulting in protein prenylation and farnesylation with classic examples seen in FDFT1. The promiscuous and overlapping functions of FDFT1 and its role outside the lanosterol pathway have made it not be considered as an actual enzyme of cholesterol biosynthesis. FDFT1 and other enzymes that have a role outside the cholesterol pathway are called the branch point enzymes of the pathway [21–23]. FDFT1 is mainly a precursor of many non-sterol products like isoprenoids and farnesylated proteins [24, 25]. Multiple studies have also demonstrated the pharmacotherapeutics potentials of statins which are inhibitors of HMGCR with few side effects. Many experimental studies have been done to seek an alternative to HMGCR inhibitors or evolving other known regulatory mechanisms beyond HMGCR in controlling cholesterol synthesis. Still, unfortunately, many of them have been met with side effects of pharmacokinetics and pharmacodynamics profile laced with high toxicity and low efficacy. So the non-statins sources of regulating cholesterol biosynthesis beyond HMGCR inhibitors, for now, have not yielded the expected results.

Here, we show for the first time that *in vitro* expression of hnRNPR in neuronal cell lines can repressively regulate the expression of HMGCR. Exquisite maintenance of cholesterol homeostasis in neuronal and non-neuronal cells is critical as dysregulation or disruption of cholesterol homeostasis can lead to a broad spectrum of pathological conditions. As a result of the critical role played by cholesterol, it is pretty germane to evolve a multifaceted and intricate entwine mechanism to keep its synthesis, metabolism, and excretion in check. The importance of this waxy substance in the pathogenesis of atherosclerosis and other cardiovascular diseases is well established and documented. Alterations in cholesterol homeostasis are not only a critical factor associated with the development of cardiovascular diseases, cancers but also responsible for a myriad of neurological and neuropsychiatric disorders such as AD, ALS, ASD, HD, NPC, PD, mood, and affective disorders which has dominated the neuroscience field lately.

2. Materials and methods

2.1 Cell culture and transfection

Neuroblastoma (N2a), MN1, and HEK293T cells originated from the ATCC and were cultured under standard cell culture conditions with DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen) at 37 °C under 5% CO_2. Cell lines were tested and found negative for mycoplasma contamination.

2.2 Plasmid transfection

hnRNPR precursor sequence was PCR amplified from N2a genomic DNA and sub-cloned into ECORV/KPN1 restriction sites of the pcDNA 3.1 (Invitrogen). After that, lentivirus was then prepared for overexpression in primary cells. The shRNA for knockdown of mouse hnRNPR experiments was cloned by obtaining the target shRNA sequences from [26]. The target shRNA sequences were sub-cloned into a pGreen Puro hairpin (H1) backbone lentivector (System Biosciences), after which lentiviruses were produced for a knockdown in primary target cells. Cloning primers were listed in **Supplementary Table 1**.

2.3 Lentiviral production

The shRNA for knockdown of mouse hnRNPR experiments was cloned by obtaining the target shRNA sequences

from [26] while the negative control shRNA was a gift from MISSION shRNA Library (Sigma, Germany). Lentivirus plasmids were transfected into 293T cells alongside the envelope plasmid (PCMV-VSV-G) and packaging plasmid (PHR'8.2deltaR). 24 hrs after transfection, the cell culture medium was replaced, and lentivirus containing medium was harvested. The virus was precipitated via ultracentrifugation and re-suspended in PBS. Viral titer was determined through qPCR. The harvested lentivirus-containing medium was then used to infect the MN1 and N2a to knockdown or overexpress hnRNPR.

2.4 RNA isolation, reverse transcription and quantitative real time PCR (qPCR)

Total RNA was isolated from cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration and quality were quantified using a spectrophotometer (Genway, UK). Using one ug of total RNA quantitative qPCR was performed with AceQTM qPCR SYBR Green Master Mix (Vazyme, China), in the presence of needed primers, Light Cycler® 96 system (Roche, Germany), using GAPDH as a control for normalization according to manufacturer's protocols. qPCR detection primers were listed in **Supplementary Table 1**.

2.5 RNA immunoprecipitation

Cultured N2a cells were harvested and lysed in PBS, plus 1% Triton-X (Sigma, Germany). The lysed cells were then ultra-sonicated for 4 min (Ningbo Scientz Biotechnology, China). The homogenates were incubated on ice for 20 min and centrifuged at 14000 rpm; the protein concentration of the supernatant was quantified by BCA protein assay (ThermoFisher Scientific, USA). An equal amount of lysates were incubated with protein A/G beads pre-conjugated with hnRNPR flag-tagged antibody or control IgG. Total RNA was isolated from the beads and subjected to qPCR analyses.

2.6 Western blot and densitometry analysis

Cells were washed twice in PBS and lysed in 1% TritonX 100 containing PBS plus a cocktail of protease inhibitors (Target MOL-EDTA free, USA). The lysates were harvested into Eppendorf tubes and subjected to ultra-sonication for 4 min and then incubated on ice for 20 min. The lysates were then centrifuged using a ThermoFisher scientific centrifuge (USA) at a speed of 14000 rpm for 10 min and 4 °C. The supernatant was then pipetted from the pellet into a new Eppendorf tube. The protein level was determined using a BCA protein Assay kit (ThermoFisher Scientific, USA) after which Protein loading buffer (250 mM Tris-HCl (5%), pH 6.8, glycerol (50%), β-mercaptoethanol (5%), bromophenol blue (0.5%), and 10% SDS) was added to the supernatant, and then heat denatured by boiling at 100 °C for 7 min at constant temperature metal bath. 20 μg of protein was then loaded into the well for immunoblotting and was resolved by SDS polyacrylamide gel electrophoresis (PAGE) gels (10–15%) and transferred onto a nitrocellulose membrane. Membranes were blocked for one and half hours using 5% non-fat dry milk diluted in TBST (pH 7.4) and probed with primary antibodies in a low-speed rocker overnight at 4 °C. Membranes were washed with TBST five times for five minutes and probed with secondary antibodies, anti-rabbit (Proteintech, USA) for one and half hours at room temperature in a slow speed rocker platform. Membranes were rewashed with TBST five times, five minutes, and protein bands were visualized using enhanced chemiluminescence (ECL Plus Detection Kit; Invitrogen, USA), and bands were detected using ChemiScope 5300 (Clinx Science Instrument, China). Primary antibodies used and their dilutions were listed in Table S2. Densitometric analyses of the immunoreactive bands were carried out using ImageJ software (National Institutes for Health, Bethesda, MD, USA).

2.7 Amplex red cholesterol assay

The relative level of cholesterol was determined via the Amplex red enzyme assays protocol (Invitrogen, USA) according to the manufacturer's instructions.

2.8 Cholesterol rescue assay

Rescue assay for the restoration of repressed cholesterol in hnRNPR overexpression was performed as MN1 and N2a cells were cultured in 6 cm dish and transfected with hnRNPR overexpression plasmids, and 48 hrs later, the cloned HMGCR plasmid construct and control pcDNA (3.1) was introduced, and 24 hrs later, cells were lysed, sonicated, and quantified after which amplex red cholesterol enzyme assays were performed following manufacturer's protocol.

2.9 Half-life assay for mRNA stability

Neuroblastoma cells (N2a) and MN1cells were cultured in a 3.5 cm dish and transfected with hnRNPR overexpression plasmids and lentivirus medium expressing hnRNPR-shRNA plasmid for 48 h. actinomycin D (ACTD) (25 μM) was used to inhibit transcription. Total RNA was isolated at different time points, i.e., 0, 2, 4, 6, and 8 h, followed by measuring the mRNA decay rates by qRT-PCR using primers specific for HMGCR. Data were plotted as fold difference over time 0 h.

2.10 Luciferase reporter assay

Luciferase assay was performed by PCR amplification of full length of WT or mutant 3′ UTR of HMGCR and inserted into Nhe1/Xba1 restriction sites of pmirGLO dual-luciferase vector (Promega) following manufacturer's instructions. N2a and MN1 cells were passed and transfected with control or hnRNPR knockdown and overexpression plasmid, 24 h later, each luciferase construct was introduced into the cells. Cells were harvested 48 h post-transfection and luciferase activity was measured by incubating cell lysates with luciferase substrate (Promega, USA). Generated data were analyzed and presented as relative fold change over control.

2.11 Statistical analysis

Images of western blot were captured using ChemiScope 5300. The area measuring tool of Image J (National Institutes of Health, Bethesda, MD, USA) was used for densito-

metric analyses of immune-reactive bands by using GAPDH-reactive bands as the normalizing loading control. GraphPad Prism v7.00 (GraphPad Software, San Diego California USA, www.graphpad.com/scientific-software/prism/) was used to plot the figures. It analyzed the data using Student's unpaired—*t*-tests to compare two groups and ANOVA for comparing more than two groups, followed by Tukey's multiple comparisons tests. In all experiments, n indicates the number of individual experiment, and each experiments is replicated three times. Data were presented as means ± SEM, whereas P-value < 0.05 was taken as statistically significant (indicated by an asterisk in the figures), $P < 0.01$ (indicated by two asterisks in the figures), $P < 0.001$ (indicated by three asterisks in the figures).

3. Results

3.1 hnRNPR overexpression and knockdown robustly decreases and enhances neuronal cholesterol, respectively

Considering the considerable physiological importance of hnRNPs in sterol homeostasis, we investigated the effects of hnRNPR on cellular cholesterol levels. Dysfunctional cholesterol metabolism results in functional and structural central nervous (CNS) pathologies and diseases such as Niemann-Pick type C disease, Huntington's disease, AD, and PD. To investigate whether hnRNPR affects neuronal function by mediating cholesterol metabolism in neuronal cells, we cultured and transfected hnRNPR overexpression and control plasmids in neuro-2a and MN1 cells. Cells were harvested 48 h post-transfection to isolate total RNA to analyze hnRNPR mRNA expression in both cell lines (Fig. 1A), which showed a substantial overexpression of hnRNPR. We then analyzed the protein level of hnRNPR in overexpression samples vs. control samples by western blot experiment. Western blot showed a significant increase in hnRNPR protein in overexpression samples compared to control in N2a and MN1, respectively (Fig. 1B). This result is also consistent with the densitometric quantification of the blots. Next, we quantified the relative cellular cholesterol level in hnRNPR-overexpression cells lysate vs. control lysate using Amplex® Red Enzyme assays (Invitrogen). The cholesterol assay revealed that the overexpression of hnRNPR substantially reduces the neuronal cholesterol levels in the two cell lines assessed (Fig. 1C). Similarly, we proceeded to investigate the effects of hnRNPR knockdown on cholesterol using the same cell lines (N2a and MN1). To ascertain how hnRNPR knockdown affects cholesterol levels and metabolism we carried out an *in vitro* knockdown of hnRNPR in the cell lines. hnRNPR knockdown plasmid (shRNA) was transfected to cells by using a lentivirus delivery system. 72 h post-transfection, cells were harvested to check the mRNA level of hnRNPR. The qRT-PCR analysis showed a robust knockdown of hnRNPR in knockdown samples compared to control (Fig. 1D). Next, we analyzed the protein level of hnRNPR by western blot. Western blot results indicated a significant knockdown of hnRNPR protein (Fig. 1E). Densitometric analysis of the knockdown blot corroborated these findings. Next, we analyzed the cellular cholesterol level in the knockdown cell lysate. The results indicate that hnRNPR knockdown significantly increases the cellular cholesterol levels in almost equimolar proportion in both cell lines that are almost impossible to distinguish between the two (Fig. 1F). Taken together, these results demonstrated that overexpression and knockdown of hnRNPR robustly decrease and increases the relative cholesterol level in neuronal cell respectively.

3.2 hnRNPR is a negative regulator of HMGCR in vitro

Maintaining neuronal cholesterol homeostasis is quite pertinent as alteration or disruption leads to an avalanche of harmful health consequences. Multiple studies have shown that changes in cholesterol homeostasis lead to a flurry of CNS disorders. We then examined how overexpression or knockdown of hnRNPR will affect or regulate enzymes involved in the cholesterol biosynthetic pathway. Although the specific binding site of hnRNPR is still unknown using the predicted binding sequence of hnRNPR as predicted by Kim *et al.*, 2005 [27] (Accession No-AY184814), with the aid of the Ensembl genome browser, the following cholesterol biosynthetic genes were identified: CYP51A1, FDFT1, HMGCR, NSDHL, and were subsequently screened. To analyze the effects of hnRNPR on the levels of the screened cholesterol biosynthetic enzymes, we transfected neuroblastoma (N2a) and MN1 cells with hnRNPR-overexpression plasmids by using the polyethylenimine (PEI) delivery method. The overexpression of hnRNPR resulted in decreased HMGCR mRNA levels of both cell lines (Fig. 2A) and protein levels of HMGCR. Still, the other binding partners of HMGCR remain unchanged (Fig. 2B), and this result is consistent with the densitometric analysis of the blots. Intriguingly and as expected the qPCR analysis to examine the mRNA levels of cholesterol enzymes (Fig. 2C) and western blot analysis to check the protein (Fig. 2D) levels of HMGCR and its binding partners using a knockdown sample of hnRNPR and probed with their respective antibodies resulted in upregulation of HMGCR and unchanged levels of binding partners in both N2a and MN1 cells and this result is in tandem with the densitometric analysis. Judging from the result (Fig. 1C) where overexpression of hnRNPR resulted in cholesterol repression, and its knockdown upregulates HMGCR, meaning that the regulation of hnRNPR is cholesterol-dependent, we ask whether supplementation of HMGCR into the suppressed hnRNPR overexpression sample will rescue this deficit via a rescue assay. Interestingly, HMGCR supplementation rescued or restored the HMGCR levels in both cell lines as there is a substantial increase in the level of HMGCR compared to the control (Fig. 2E). Overall, these findings suggest that KD or OV of hnRNPR enhances and represses HMGCR mRNA and protein levels respectively but has no effect on binding partners of HMGCR and the repressed cholesterol by hnRNPR overexpression was rescued via a rescue assay.

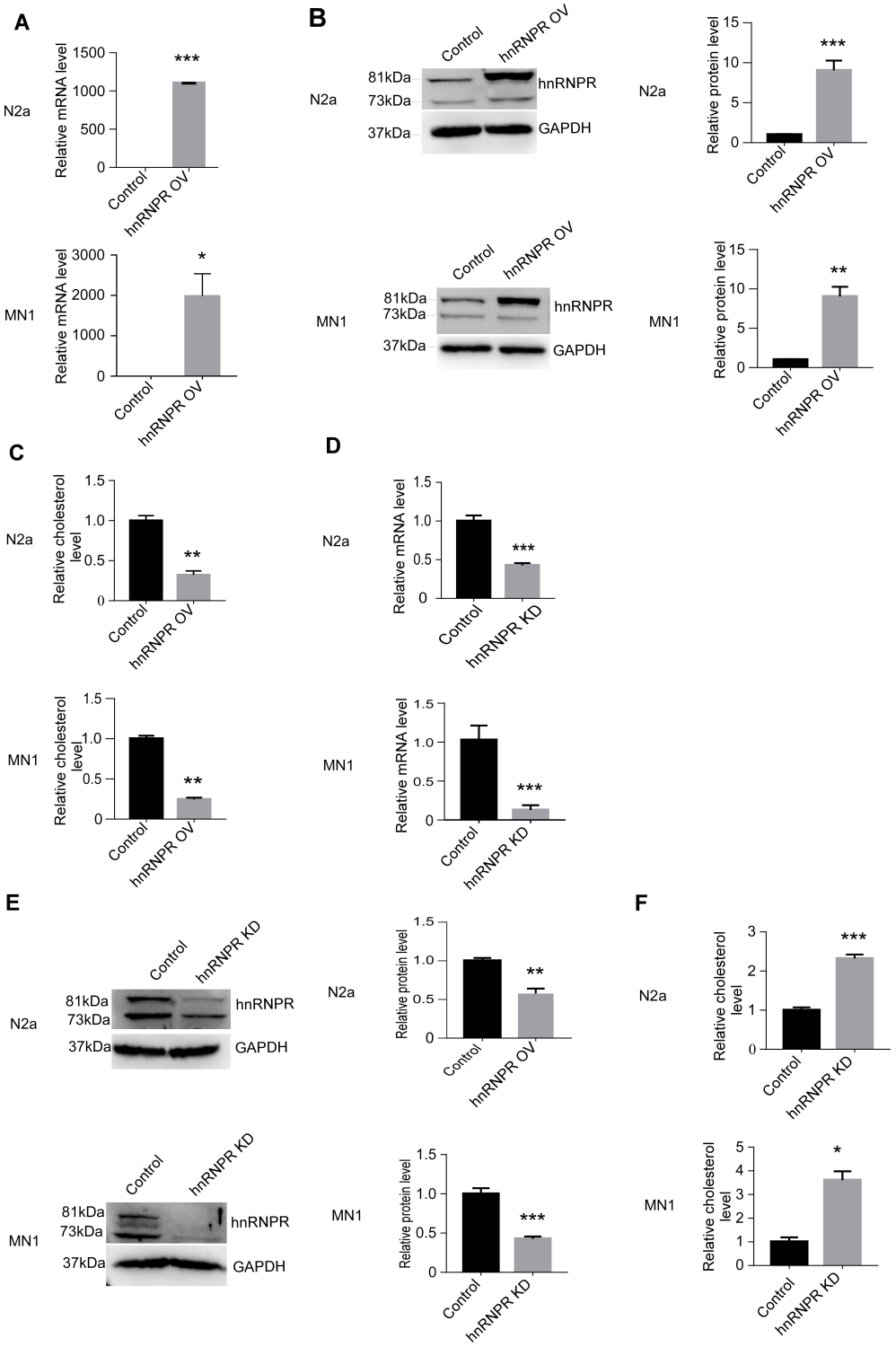

Fig. 1. hnRNPR overexpression and knockdown robustly decrease and enhances cholesterol level, respectively. (A) The relative mRNA levels of hnRNPR in control or hnRNPR overexpression N2a and MN1 cells, assessed by qPCR analysis. Data are presented as a fold change of overexpression over control. GAPDH was used as a loading control, n = 3. (B) Protein level of hnRNPR in control or overexpression hnRNPR as assessed by western blot in both cell lines of N2a and MN1 and their densitometric analysis. GAPDH was included as a loading control, n = 3. (C) Relative cholesterol level normalized to protein level as determined by Amplex red enzyme assay in N2a and MN1 cells. Data plotted as the relative level of control over hnRNPR-overexpression. (D) The relative mRNA levels of deficient hnRNPR in control or hnRNPR KD in N2a and MN1 cells, assessed by qPCR analysis. Data are presented as a fold change of overexpression over control. GAPDH was used as a loading control, n = 3. (E) Protein level of hnRNPR in control or deficient hnRNPR in both cell lines as assessed by western blot with GAPDH included as a loading control, n = 3. Also included is the densitometric analysis from western blot. (F) Relative cholesterol level normalized to protein level as determined by Amplex red enzyme assay in knockdown of N2a and MN1 cells. For this and subsequent figures, OV, denotes Overexpression, KD, Knockdown. $^{**}P < 0.01$; $^{***}P < 0.001$ by Student's *t*-test; error bars denote means ± SEM.

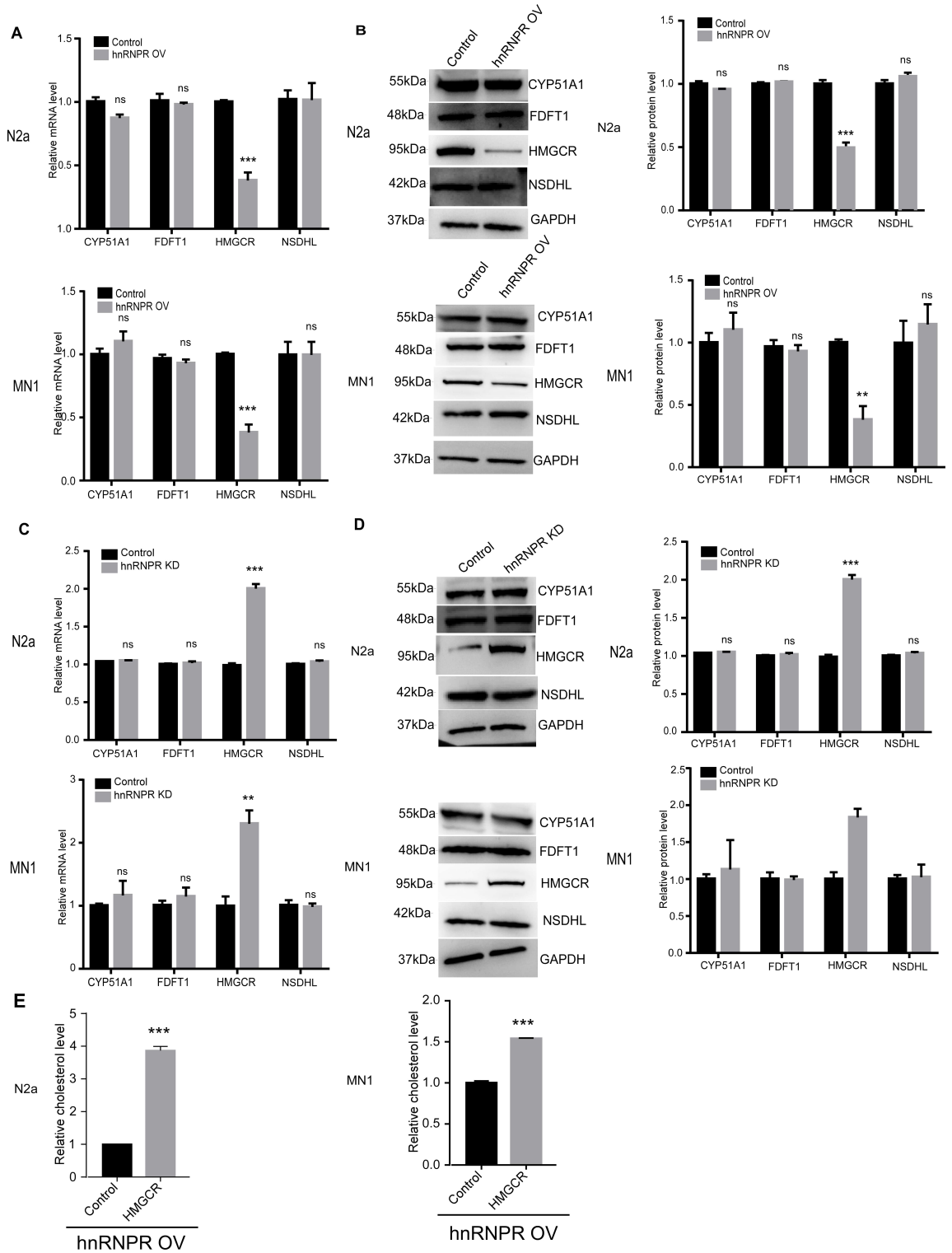

Fig. 2. hnRNPR is a negative regulator of HMGCR *in vitro*. (A) The relative mRNA levels of cholesterol biosynthetic genes in cultured N2a and MN1 cells of overexpression hnRNPR were assessed by qPCR analysis. GAPDH was used as internal control, n = 3. (B) Western blot to examine the protein levels of cholesterol enzymes in hnRNPR overexpression or control of both cell lines. GAPDH was included as a loading control, n = 3. Also included is the densitometric analysis (from panel B), consistent with the western blot. (C) The relative mRNA levels of cholesterol biosynthetic enzymes in cultured N2a and MN1 cells knockdown or control assessed by qPCR analysis. GAPDH was used as internal control, n = 3. (D) Levels of cholesterol enzymes in cultured N2a and MN1 were determined by western blot, GAPDH was included as a loading control, n = 3. Densitometric analysis of WB from panel D was also included. (E) Relative cholesterol level normalized to protein level as determined by Amplex red enzyme assay in N2a and MN1 cells to determine the rescue assay in repressed hnRNPR OV. Data plotted as the relative level of control over hnRNPR-overexpression. *** $P < 0.001$ by Student's *t*-test; ANOVA; followed by Tukey's multiple comparisons tests; error bars denote means ± SEM.

3.3 hnRNPR binds to 3′ UTR of HMGCR and stabilizes its mRNA level

Being an RNA binding protein, hnRNPR possesses three RRM with well-conserved RNP-1 and RNP-2 sub-motifs with which it could base pair with 3′ UTR of HMGCR mRNA by direct binding. We cultured N2a cells and subjected the lysates to immunoprecipitation using a flag tag antibody to prove this hypothesis. Our result indicated a successful immunoprecipitation as detected by western blot (Fig. 3A). The RIP assay followed the IP assay. The cell lysate was incubated with a flag-tag antibody to immune-precipitate the complexes. We then followed up with qPCR analysis, which revealed a significant fold enrichment of HMGCR mRNA compared to control IgG, and no significant difference was observed for GAPDH mRNA (Fig. 3B). To further prove that hnRNPR is a regulatory target for HMGCR, we next sought to ascertain how hnRNPR affects mRNA stability. To determine the mRNA stability, we cultured N2a, MN1 cells and transfected them with an hnRNPR overexpression sample and shRNA for KD using a lentiviral mediated delivery system. We then decided to determine the mRNA half-life of HMGCR by treating MN1 and N2a cells with a transcription inhibitor, ACTD, and analyze mRNA levels at various time points after the treatment. Interestingly, our findings revealed shorter mRNA half-lives in overexpression samples of both cell lines (Fig. 3C) and longer mRNA half-lives in knockdown samples (Fig. 3D). Collectively, these data indicated that hnRNPR binds directly to HMGCR mRNA and subsequently altered its level.

3.4 hnRNPR binds directly to 3′ UTR of HMGCR and represses its expression level

Being an RNA-binding protein, a myriad of potential roles of hnRNPR in splicing, transport of RNAs, and regulation of RNA stability have been reported through binding to the 3′ UTR of mRNA. Having known that hnRNPR contains the predicted binding site to 3′ UTR of HMGCR, as shown in the schematic (Fig. 4A), we proceeded to evaluate whether hnRNPR can bind directly to the 3′ UTR of HMGCR mRNA using these sequences. Therefore, we provided a luciferase reporter construct that harbors the full length of 3′ UTR of HMGCR alongside the mutant 3′ UTR by sub-cloning each putative hnRNPR binding site and 500 bp upstream and downstream sequences into PGK promoter containing pmirGlo luciferase vector. Construct containing the wild-type or mutant 3′ UTR were inserted into the control or hnRNPR overexpression N2a and MN1 cells. Luciferase assay result indicated that HMGCR wild-type compared to control showed reduced luciferase activities of the HMGCR-WT in 3′ UTR of both cell lines (Fig. 4B), while the comparison of HMGCR WT to HMGCR-mut indicated increased activity of the HMGCR-mut (Fig. 4C).

On the contrary,hnRNPR knockdown cells introduced into the control compared to HMGCR-wild-type showed a substantial increase in luciferase activity of HMGCR-wild-type in both cell lines (Fig. 4D). Comparison of HMGCR-WT to HMGCR-mut showed a decreased luciferase activity of the HMGCR-mut in hnRNPR deficient cells introduced with mutant 3′ UTR in both N2a and MN1 cells (Fig. 4E). Together, these data suggest that hnRNPR binds directly to the 3′ UTR of HMGCR and represses its expression. The overall working model was demonstrated in Fig. 5.

4. Discussion

Perturbations in the homeostatic regulation of neuronal and non-neuronal cholesterol are precursors for many cardiovascular and neurological disorders. Therefore tight regulations of this waxy substance are essential to prevent these pathological insults. The roles of hnRNPR in neuronal development, pre-mRNA processing of β-actin mRNA, and its appropriate movement to the growth cone of the developing motor neuron and its role in neurological disorders are well established [26, 28]. Also recently, Duijkers and his co-researchers corroborated the neuronal and non-neuronal developmental disorder of hnRNPR by investigating hnRNPR variants that impair expression of the homeobox gene and how it accentuate developmental disorders in human. Multiple works from several groups have reported the roles of RNA-binding proteins in modulating cholesterol homeostasis and lipid metabolism through transcriptional and post-transcriptional processes. More importantly, dysregulation of RBPs expression has been associated with neurological pathologies, suggesting that manipulating these RBPs could be a valuable therapeutic target in drug design and development aimed at mitigating the attendant neurological diseases. However, despite the aforementioned plethora and pleiotropic roles and functions attributed to hnRNPR, little is known about its roles in regulating neuronal cholesterol metabolism. Although the role of heterogeneous nuclear ribonucleoprotein (hnRNPs), the most prominent family of RBPs in cholesterol metabolism and modulation specifically, the regulation of HMGCR, has been previously reported by [29]. The dual role of hnRNPA1 in cholesterol modulation and alternative splicing of HMGCR was demonstrated that hnRNPA1 modulates cholesterol in a twin manner.

On the one hand, through the alternatively spliced variant of HMGCR that lacks exon 13 (-13), which mimics statin, an HMGCR degrader as it potentiates the production and degradation of the variant, thereby increases LDL-C uptake as the activity of the HMGCR enzyme is waned paving the way for increased LDL-C uptake and decreased plasma cholesterol level. Since the variation of statin response, primarily reduction in LDL-C with statin treatment is mediated through HMGCR (-13), which acts as a marker and determinant of statin response. It suggests that hnRNPA1 plays a role in the variation of response to cardiovascular disorder. By this process, it alleviates cholesterol-mediated or cardiovascular disorders. The regulation of the alternatively spliced variant of HMGCR (-13) occurred when hnRNPA1 binds directly to the HMGCR transcript, and HMGCR SNP rs384666 augments this interaction in a genetically dependent manner.

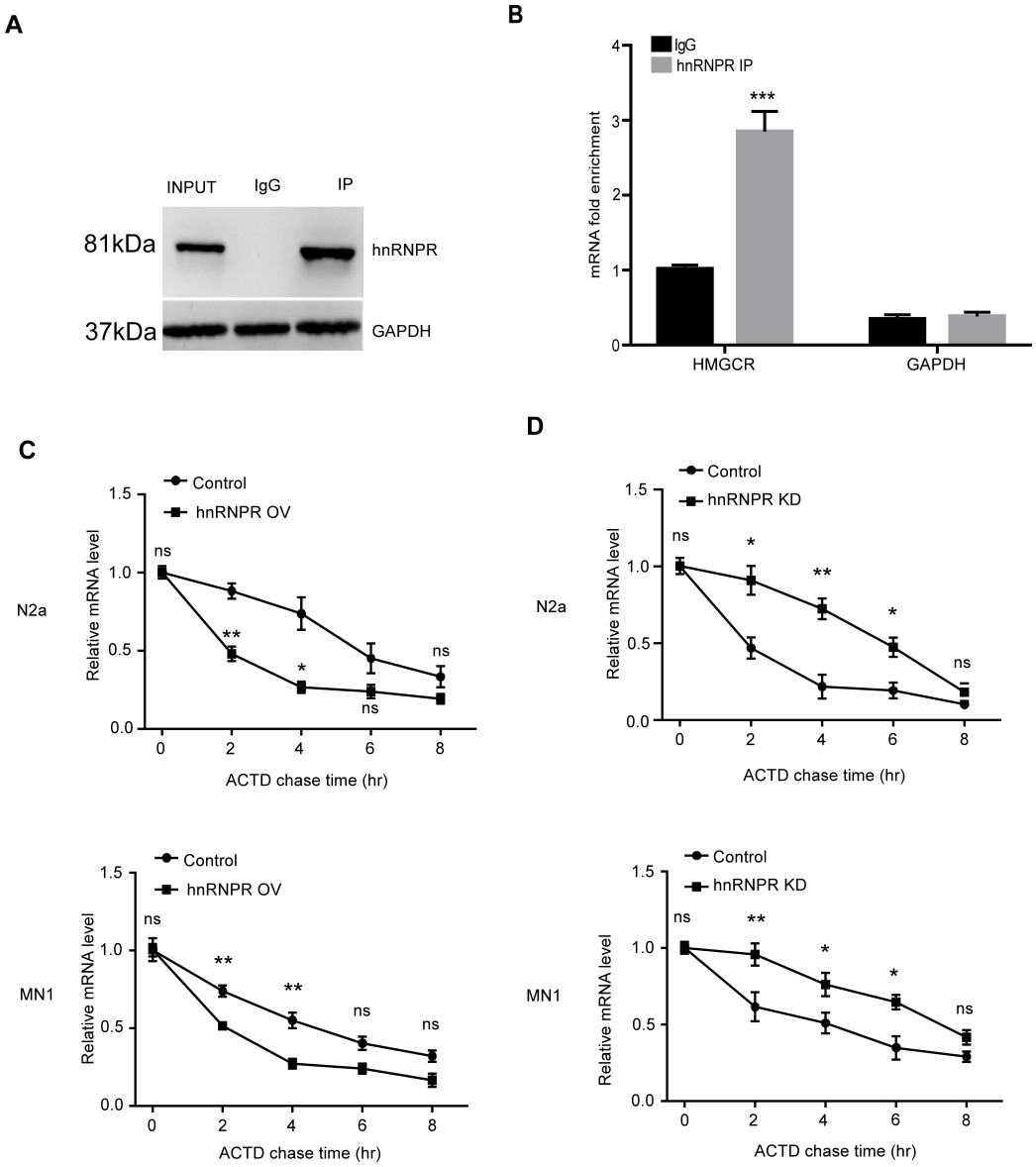

Fig. 3. hnRNPR binds to 3′ UTR of HMGCR and stabilizes its mRNA level. (A) N2a cells were subjected to immunoprecipitation with hnRNPR flag-tag antibody or control IgG and GAPDH followed by immunoblotting with a flag-tag antibody. (B) Total RNA was isolated and subjected to qPCR analysis. Data are shown to enrich the hnRNPR flag-tag antibody over control IgG in triplicate (n = 3). GAPDH was included as a negative control. (C) Relative mRNA of HMGCR in hnRNPR overexpression and control N2a and MN1 cells subjected to 25 uM of actinomycin D. (D) Relative mRNA of HMGCR in hnRNPR knockdown and control N2a and MN1 cells subjected to 25 uM of actinomycin D. Data were plotted as fold difference over 0 hours. $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's *t*-test; ANOVA; followed by Tukey's multiple comparisons test; error bars denote means ± SEM.

Apart from hnRNPA1 stimulating the production of HMGCR (-13) transcript, it also avidly and preferentially stabilizes HMGCR (-13) over HMGCR with intact exon 13 (HMGCR +13). The subcellular localization of hnRNPA1 in the cytoplasm and nucleus is responsible for this double-edged effect of transcript stability and alternative splicing, respectively. It is also possible that the effects of cholesterol metabolism of transcript stability and alternative splicing by hnRNPA1 may transcend beyond HMGCR, judging from the fact that hnRNPA1 has been shown to interact with another critical enzyme involved in lipid metabolism as seen in APOE. Unlike the hnRNPA1 that uses the alternatively spliced variant of HMGCR -13, which always predominates over HMGCR +13 in alternative splicing and cholesterol modulation. Here, we report for the first time that hnRNPR, an essential but less-studied member of the RBPs, acts as a novel post-transcriptional regulator of HMGCR expression using *in vitro* culture technique. Just like it is also possible that the effects of cholesterol metabolism of transcript stability and alternative splicing by hnRNPA1 may transcend beyond HMGCR judging from the fact that hnRNPA1 has been shown to interact with another critical enzyme involved in lipid metabolism as seen in APOE, this phenomenon may also likely apply to hnRNPR. We identify hnRNPR as a negative

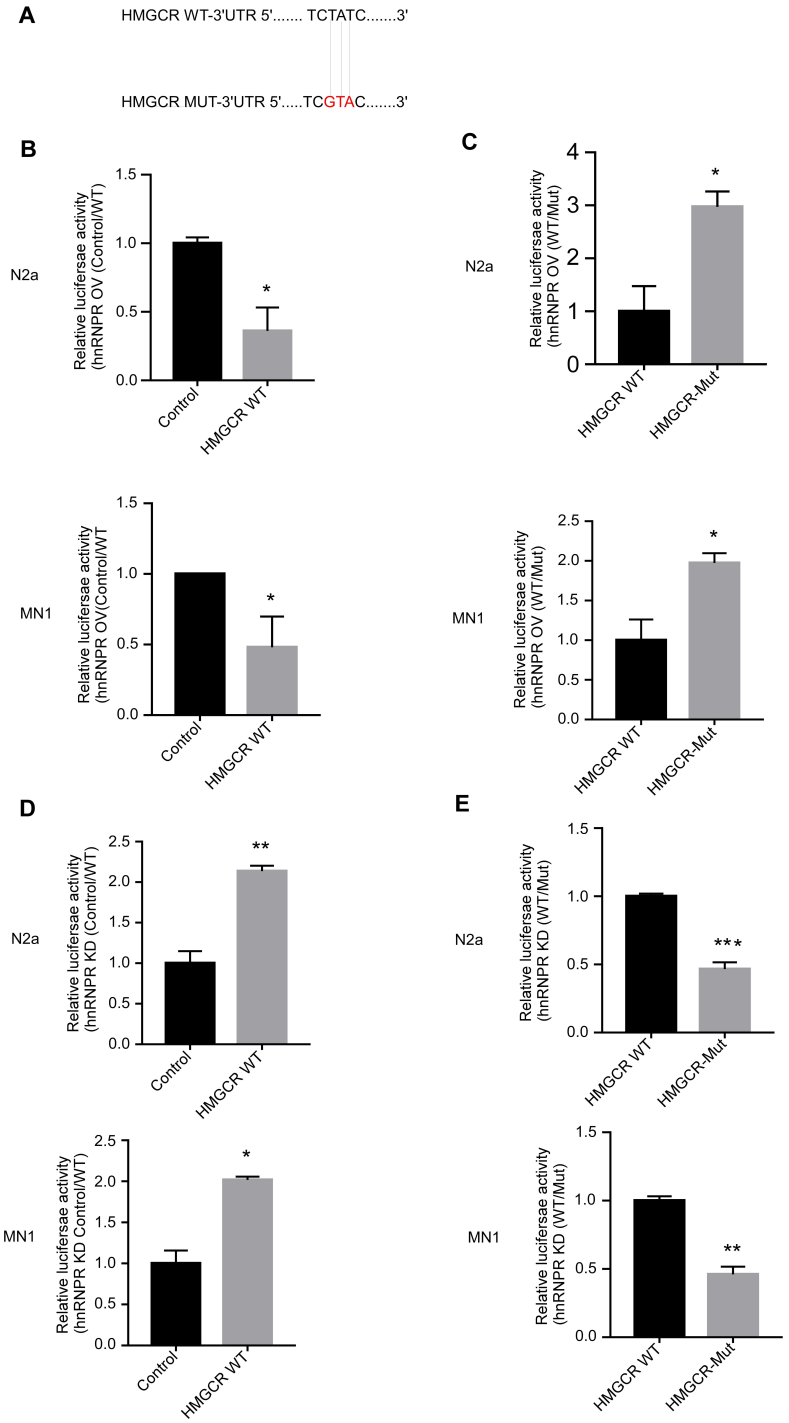

Fig. 4. hnRNPR binds directly to the 3′ UTR of HMGCR and represses its expression level. (A) Schematic representation of hnRNPR binding sequence, predicted binding sequence of 3′ UTR of HMGCR containing the mutation. (B) 3′ UTR of HMGCR WT cloned into control pmirGlo luciferase vector and transfected into N2a and MN1 cells of hnRNPR overexpression. Luciferase assay determined binding affinities, which showed a decreased luciferase of the HMGCR WT compared to control. Luciferase activity values were presented as relative level to control, n = 3. (C) 3′ UTR of HMGCR WT, 3′ UTR of HMGCR mutant were cloned differently into pmirGlo luciferase vector and transfected into N2a and MN1 cells of hnRNPR overexpression. Binding affinities were determined by luciferase assay. HMGR WT is used to compare with HMGCR-mut, indicating a higher luciferase activity than HMGCR WT. Luciferase activity values were presented as relative level to control, n = 3. (D) 3′ UTR of HMGCR WT cloned into pmirGlo luciferase vector and transfected into N2a and MN1 cells of hnRNPR KD. Binding affinities were determined by luciferase assay. Luciferase activity values were presented as relative level to control, n = 3. (E) 3′ UTR of HMGCR WT, 3′ UTR of HMGCR mutant were cloned differently into pmirGlo luciferase vector and transfected into N2a and MN1 cells of hnRNPR KD. Binding affinities were determined by luciferase assay. HMGR WT is used as a control to compare with HMGCR-mut. Luciferase activity values were presented as relative level to control, n = 3. WT, Wild type; MUT, Mutation; ***$P < 0.001$; by ANOVA; followed by Tukey's multiple comparisons tests; error bars denote means ± SEM.

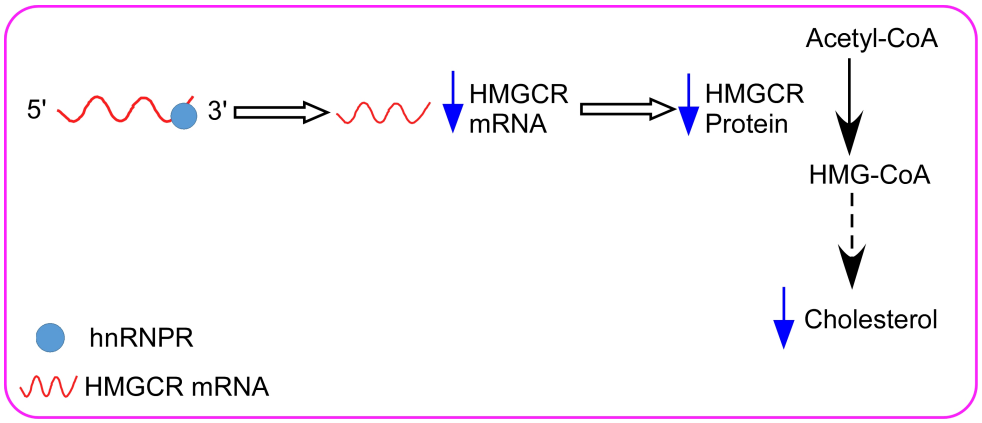

Fig. 5. hnRNP R reduces cholesterol levels via suppressing HMGCR expression.
HnRNPR binds to the 3′ UTR of HMGCR mRNA and destabilized HMGCR mRNA. Decreased HMGCR levels lead to decreased production of cholesterol.

regulator of HMGCR when it is overexpressed in neuroblastoma and MN1 cell lines. There was a significant decrease in both mRNA and protein levels of HMGCR compared to the control (Fig. 2A,B). Tight regulation of brain lipid, especially cholesterol being the primary lipid in the brain, is crucial as dysfunctions in neuronal cholesterol homeostasis have been extensively related to brain pathology like AD. Neuronal cholesterol alone accounts for approximately 25% of the body's total unesterified cholesterol, and approximately 70% is found in the myelin sheath, with the rest found in glial and neuronal membranes [30]. Cholesterol is an essential constituent for the normal functioning of the nervous system, and plays an essential role during the developmental stage and adult life [31].

Here, we report that hnRNPR knockdown substantially upregulates relative cholesterol (Fig. 1F), HMGCR mRNA, and protein levels, which could modulate cholesterol levels by elevating it towards homeostatic and physiological range hypocholesterolemic state. Despite the importance of neuronal cholesterol, excess of it is highly detrimental to brain health and function. Just as a decrease in neuronal cholesterol results in diverse neuropathology, it is also in excess. Transcriptionally activated HMGCR by sterol- regulatory-element binding protein 2 (SREBP-2) binding to its promoter region increases cholesterol synthesis and turnover, and it is already established that the isoform 4 (ε4) of the cholesterol transport protein apolipoprotein E is a significant risk factor for AD development [32]. *In vitro* and *in vivo* studies also demonstrated the potential of decreasing Alzheimer's disease prevalence by administering two cholesterol synthesis inhibitors of lovastatin and pravastatin [33, 34]. Similarly, multiple studies reported a link between hypercholesterolemia and its propensity to increase brain Aβ immunoreactivity in rabbits [35–40]. Overexpression of hnRNPR exquisitely decreases cholesterol (Fig. 2B), exemplified HMGCR degrader or inhibitor as it decreases 3-hydroxy-3-methylglutaryl-CoA reductase mRNA and protein levels (HMGCR), an enzyme needed to reduce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) towards mevalonate with the ultimate goal of cholesterol synthesis, by this degradative action of overexpression hnRNPR, cholesterol level is decreased which could prevent hypercholesterolemia. Overexpression of hnRNPR here mimics statins which are competitive inhibitors of HMGCR.

Our findings provide insights on the possibility of overexpression of hnRNPR to homeostatically regulate cholesterol within the normal physiological range under hypercholesteremic state. And to date, no prophylactic or curative drug has been able to usurp HMG CoA reductase inhibitors from being dubbed the most widely prescribed and well-tolerated lipid-lowering drugs in use. All these studies are a pointer that HMGCR regulating cholesterol is indirectly implicated in the vicious cycles of AD pathology, other neurodegenerative diseases and cardiovascular disorders. hnRNPR possesses three RRM with well-conserved RNP-1 and RNP-2 sub motifs [41]. The C terminus also contains an RGG box used to collaborate with RRMs for RNA recognition and binding specificity. To ascertain the binding potentials of hnRNPR to HMGCR mRNA, we performed IP and RIP assays. hnRNPR lysates were incubated with flag-tag antibody, and the resultant bounded hnRNPR RNA was then subjected to qPCR analyses. Our result indicated a successful pulldown of HMGCR as demonstrated by western blot and mRNA fold enrichment, respectively (Fig. 3A,B). The regulatory and repressive effects of hnRNPR on HMGCR were further demonstrated via the luciferase reporter assay (Fig. 4A,B,C,D,E). We also demonstrate a schematic representation (Fig. 5) depicting the interplay of the cholesterol biosynthetic genes regulated by hnRNPR.

Taken together, our findings provide novel evidence that hnRNPR is involved in neuronal cholesterol metabolism, suppresses cholesterol by inhibiting HMGCR in cultured N2a and MN1 cells.

5. Conclusions

In conclusion, modulation of neuronal cholesterol by hnRNPR could be a valuable tool in health and disease states. It

provides insights into understanding AD's physiology, pathogenesis and other diverse neuropathological disorders, cancers and cardiovascular diseases, where cholesterol dyshomeostasis has been implicated. These data suggest that modulation of cholesterol by hnRNPR could open new vistas in understanding the physiology, pathogenesis, and pharmacotherapy of cholesterol related disorders caused by dysfunction of cholesterol homeostasis.

Abbreviations

3′ UTR, 3′ untranslated region; ACTD, actinomycin D; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ASD, autism spectrum disorder; CYP51A1, lanosterol 14 α-demethylase; FDFT1, farnesyl diphosphate farnesyltransferase1; FTLD, frontotemporal lobar degeneration; HD, Huntington's disease; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; hnRNPR, heterogeneous nuclear ribonucleoprotein R; hnRNPs, heterogeneous nuclear ribonucleoproteins; NPC, Niemann-Pick type C disease; NSDHL, NAD(P)H steroid dehydrogenase like protein; PD, Parkinson's disease; RBPs, RNA binding proteins; RRM, RNA recognition motif.

Author contributions

QL supervised the project; JA, JZ, and QL conceived, designed and conceptualized the research; and JA performed experiments; JA, JZ, ARA, DL, QZ, XL, WL, analyzed data; JA, JZ and QL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://jin.imrpress.com/EN/10.31083/j.jin2002026.

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