International Journal of Neuropsychopharmacology (2020) 23(4): 257–267

doi:10.1093/ijnp/pyaa014
Advance Access Publication: 2 March 2020
Regular Research Article

REGULAR RESEARCH ARTICLE

Upregulation of TRPC6 Mediated by *PAX6* Hypomethylation Is Involved in the Mechanical Allodynia Induced by Chemotherapeutics in Dorsal Root Ganglion

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Abstract

Background: Although the action mechanism of antineoplastic agents is different, oxaliplatin, paclitaxel, or bortezomib as first-line antineoplastic drugs can induce painful neuropathy. In rodents, mechanical allodynia is a common phenotype of painful neuropathy for 3 chemotherapeutics. However, whether there is a common molecular involved in the different chemotherapeutics-induced painful peripheral neuropathy remains unclear.

Methods: Mechanical allodynia was tested by von Frey hairs following i.p. injection of vehicle, oxaliplatin, paclitaxel, or bortezomib in Sprague-Dawley rats. Reduced representation bisulfite sequencing and methylated DNA immunoprecipitation were used to detect the change of DNA methylation. Western blot, quantitative polymerase chain reaction, chromatin immunoprecipitation, and immunohistochemistry were employed to explore the molecular mechanisms.

Results: In 3 chemotherapeutic models, oxaliplatin, paclitaxel, or bortezomib accordantly upregulated the expression of transient receptor potential cation channel, subfamily C6 (TRPC6) mRNA and protein without affecting the DNA methylation level of TRPC6 gene in DRG. Inhibition of TRPC6 by using TRPC6 siRNA (i.t., 10 consecutive days) relieved mechanical

Received: October 24, 2019; **Revised:** February 1, 2020; **Accepted:** February 25, 2020

Significance Statement

Activation of PAX6/TRPC6 in DRG neurons may be a common signal pathway for mechanical allodynia following oxaliplatin, paclitaxel, or bortezomib application. A DNA hypomethylation-mediated epigenetic mechanism in the PAX6 gene contributed to the upregulation of the PAX6/TRPC6 pathway. Targeted on PAX6/TRPC6, signaling may provide a new idea and reliable basis for the treatment of different chemotherapeutics-induced chronic pain.

allodynia significantly following application of chemotherapeutics. Furthermore, the downregulated recruitment of DNA methyltransferase 3 beta (DNMT3b) at paired box protein 6 (PAX6) gene led to the hypomethylation of PAX6 gene and increased PAX6 expression. Finally, the increased PAX6 via binding to the TPRC6 promoter contributes to the TRPC6 increase and mechanical allodynia following chemotherapeutics treatment.

Conclusions: The TRPC6 upregulation through DNMT3b-mediated PAX6 gene hypomethylation participated in mechanical allodynia following application of different chemotherapeutic drugs.

Key Words: Chemotherapeutics, TRPC6, PAX6, mechanical allodynia, DNA methylation

Introduction

In spite of their different antitumor mechanisms, any one of the chemotherapeutic drugs oxaliplatin, paclitaxel, or bortezomib causes painful neuropathy (Liu et al., 2016; Xu et al., 2017; Meng et al., 2019). Clinically, the manifestation of painful neuropathy is varied, including tingling, loss of proprioception sense, burning pain, etc. (Haythornthwaite and Benrud-Larson, 2001; Brandolini et al., 2019). Similar to clinical manifestations, painful neuropathy is manifested in various forms in rodent models. Among these symptoms, mechanical allodynia is a common phenotype with oxaliplatin, paclitaxel, or bortezomib (Laumet et al., 2015; Huang et al., 2016; Stockstill et al., 2018). Studies showing that transient receptor potential cation channel subfamily V member 1 (TRPV1) play an important role in the burning pain in humans (Simone et al., 1989; Li et al., 2015) and thermal hyperalgesia in rats (Hara et al., 2013) suggested that TRPV1 may be a key ion channel mediating thermal pain. However, it remains unclear whether there is a common ion channel involved in the mechanical allodynia induced by different chemotherapeutic agents.

The excitability of DRG neurons is fundamentally determined by the functional activities of neuronal ion channels in various conditions of chronic pain. For example, the change of sodium channel, calcium channel, or other ion channel in DRG is involved in the chronic pain induced by nerve injury or inflammation (Fischer et al., 2017; Sakai et al., 2017; Shan et al., 2019). Mammalian transient receptor potential (TRP) channel proteins can be classified into 6 subfamilies: TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML (Ramsey et al., 2006). The TRP cation channel, subfamily C (TRPC) consists of 7 members (TRPC1–7) and serves a wide range of physiological functions ranging from proliferation of cells to mechanical sensory transduction (Nakao et al., 2015). Among them, TRPC6 upregulation contributed to chronic morphine-induced hyperalgesia (Jin et al., 2017). Furthermore, TRPC6 is also involved in the mechanical allodynia induced by inflammatory or diabetes (Alessandri-Haber et al., 2009; Roa-Coria et al., 2019). Whether TRPC6 is a common molecular to mediate the mechanical allodynia induced by different chemotherapeutics remains unclear. Studies have shown that epigenetic mechanisms such as DNA methylation play an important role in chronic pain through regulating target protein expression (Anis and Mosek, 2018; Louwies et al., 2019). However, whether DNA methylation is involved in TRPC6 expression is unknown.

PAX6, as a member of the paired box family, plays a critical role in brain development (Walther and Gruss, 1991; Wullimann and Rink, 2001). For example, PAX6 regulates the proliferation of neural stem mediate autism spectrum disorder (Kikkawa et al., 2019). As a conserved transcription factor with 2 different DNA-binding domains, PAX6 also mediates both embryonic and adult neurogenesis (Osumi et al., 2008). Furthermore, a recent study showed that adult spinal cord neurogenesis via increasing neuronal excitability contributed to chronic pain (Rusanescu, 2016). However, to date, no evidence to our knowledge has shown that PAX6 in DRG neurons is involved in chronic pain. In addition, as a transcription factor, whether PAX6 mediates TRPC6 expression remains unclear.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 200 to 220 g were obtained from the Institute of Experimental Animals of Sun Yat-sen University and housed in a temperature- and humidity-controlled room (50–60% humidity, 22°C ± 1°C) on a 12-h-light/-dark cycle. Animals were housed in separated cages with ad libitum access to food and water. All experimental protocols were approved by the Animal Care and Use Committee of Sun Yat-sen University and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and ethical guidelines.

Animals were randomly assigned to the experimental groups. Each group included more than 3 animals, and the specific number of animals in each group is indicated in the figure legends. All data were collected and analyzed by 2 independent observers who were blinded to the group assignment of the animals or the treatment group during analysis.

Drug Application and Behavioral Test

Oxaliplatin (4 mg/kg, Sigma Aldrich, St. Louis, MO), paclitaxel (8 mg/kg, Bristol-Myers Squibb, New York City, NY), and bortezomib (0.2 mg/kg, Haoran Biological Technology Co., Shanghai, China) were prepared before each treatment and injected i.p. Oxaliplatin or bortezomib was i.p. injected once a day

for 5 consecutive days, and paclitaxel was injected once a day on days 1, 4, and 7.

TRPC6 siRNA, PAX6 siRNA, or scramble siRNA (1 nmol, 10 μL, once a day) was injected intrathecally for 10 consecutive days. Intrathecal injection was in accordance with the previous method (Xu et al., 2017). In brief, a polyethylene-10 catheter was inserted into the L5/L6 intervertebral subarachnoid pace of the rat following the injection of sodium pentobarbital (50 mg/kg, i.p.), and the localization of the catheter tip was placed at the L5 spinal segmental level. After 5 days of recovery, 5 rats with hind limb paralysis or paresis after surgery were excluded from the study.

The 50% paw withdrawal threshold was assessed using von Frey hairs (Ugo Basile, Gemonio, Varese, Italy) according to our and peer's previous reports (Chaplan et al., 1994; Xu et al., 2017). In short, each animal was loosely restrained in a plastic box and allowed to adapt for at least 15 minutes per day for 3 consecutive days before testing. Then, series of von Frey filaments with different bending force were presented to the mid-plantar surface of each hind paw. In the absence of a paw withdrawal response to the initially selected hair, a stronger stimulus was presented; in the event of paw withdrawal, the next weaker stimulus was chosen. Optimal threshold calculation by this method required 6 responses in the immediate vicinity of the 50% threshold.

qPCR (Real-time Quantitative PCR Detecting System) and qPCR Array

Under anesthesia using sodium pentobarbital, L4 to L6 DRGs in rats were isolated, and TriZol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA. The reverse transcription was performed with Prime Script RT Master Mix (Takara Bio Inc., Kusatsu, Shiga, Japan). Real-time quantitative polymerase chain reaction (qPCR) was performed with SYBR Premix Ex TaqII (Takara). The relative expression ratio of mRNA expression was quantified through the $2^{-\Delta\Delta CT}$ method. Table 1 shows the primer sequences of PCR for the investigated mRNA.

For qPCR array, total RNA from DRGs was extracted using RNeasy Mini Kit (Qiagen, 74104; Qiagen, Frankfurt, Germany), and RT^2 Profiler PCR Array (Qiagen, 330401) was used to detect the changes of ion channel.

Nucleo-cytoplasmic Separation and Western Blot

The bilateral L4–L6 DRGs of rats were removed under sodium pentobarbital anesthesia (50 mg/kg dose, i.p.). Cytoplasm and nucleus protein of the DRGs were separated according to the manipulation of the NE-PER nuclear and cytoplasmic extraction reagents kit (ThermoFisher Scientific, Waltham, MA) (Nie et al., 2018). Briefly, the samples were homogenized on ice in a 15-mmol/L Tris buffer containing a cocktail of proteinase inhibitors and phosphatase inhibitors, and the concentration of protein was determined with a BCA protein assay (ThermoFisher Scientific). The protein samples were separated via gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated in a primary antibody against TRPC6 (1:200, Alomone Labs, Jerusalem, Israel), PAX6 (1:900, Abcam, Cambridge, UK), DNMT3b (1:500, Abcam), β-actin (1:1000, CST, Boston, MA), and histone H3 (1:1000, Abcam) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies were used to incubate the blots. Enhanced chemiluminescence solution (Pierce) was used to detect immunocomplexes. Each band was quantified using a computer-assisted imaging analysis system (NIH ImageJ).

Table 1. Specific Primer Sequences for qPCR

Gene	Primer	Sequence
TRPC6 (Rat)	Forward	5′- GCGGCAGACAGTTCTTCGTGAG -3′
	Reverse	5′- CTTCTAGCATCTTCCGCACCACTG -3′
DNMT1 (Rat)	Forward	5′- GACCAATGAGGCACTGTCCG -3′
	Reverse	5′- GACCGCGACTGCAATACACA -3′
DNMT3a (Rat)	Forward	5′- GGCCCATTCGATCTGGTGA -3′
	Reverse	5′- CTTGGCTATTCTGCCGTGTTC -3′
DNMT3b (Rat)	Forward	5′- GATGATCGACGCCATCAAG -3′
	Reverse	5′- CGAGCTTATCATTCTTTGAAGCTA -3′
PAX6 (Rat)	Forward	5′- CAGAGTTCTTCGCAACCTGGCTAG -3′
	Reverse	5′- GGTCTGTCCGTTCAGCATCCTTAG -3′
β-actin (Rat)	Forward	5′-AGGGAAATCGTGCGTGACAT-3′
	Reverse	5′-GAACCGCTCATTGCCGATAG-3′

Immunohistochemistry

Perfusion was performed through the ascending aorta with 4% paraformaldehyde after application of sodium pentobarbital (50-mg/kg dose, i.p.). The removed DRGs were placed into 4% paraformaldehyde for post fixing overnight. Cryostat sections of 16-μm thickness were obtained, and immunohistochemistry was performed with primary antibodies for TRPC6 (1:100; Alomone Labs), PAX6 (1:200; Abcam), NF200 (1:200; Chemicon), IB4 (1:50; Sigma Aldrich), and GFAP (1:400; CST). After overnight incubation at 4°C, the sections were incubated with secondary antibodies, which conjugated with cy3 or Alex488-fluorescein isothiocyanate for 1 hour at room temperature. A Nikon (Nikon Eclipse Ni-E, Japan) confocal microscopy was used to examine the fluorescence of tissue slices.

Reduced Representation Bisulfite Sequencing

Genomic DNA was extracted from DRG of 10 days after chemotherapeutic drug group or vehicle group using the QIAmp DNA Micro Kit (Qiagen, 56 304). Reduced representation bisulfite sequencing (RRBS) sequencing libraries were prepared as describe previously (Gu et al., 2011). Briefly, a total amount of 5.2 μg genomic DNA spiked with 26 ng lambda DNA was fragmented by sonication to 200 to 300 bp with Covaris S220 followed by end repair and adenylation. Cytosine-methylated barcodes were ligated to sonicated DNA as per manufacturer's instructions. Furthermore, these DNA fragments were treated twice with bisulfite using an EZ DNA Methylation-Gold Kit (Zymo Research) before the resulting single-strand DNA fragments were PCR amplificated using KAPA HiFi HotStart Uracil + ReadyMix (2X). Qubit 2.0 Fluorometer (Life Technologies, CA) and qPCR were used to quantify library concentration, and the insert size was assayed on an Agilent Bioanalyzer 2100 system. Then the library preparations were sequenced on an Illumina Hiseq 2500/4000 or Novaseq platform, and 125-bp/150-bp paired-end reads were generated. Finally, image analysis and base calling were performed with Illumina CASAVA pipeline, and 125-bp/150-bp paired-end reads were generated.

Methylated DNA Immunoprecipitation Assay

Rats were deeply anesthetized with sodium pentobarbital (50-mg/kg dose, i.p.), and then the L4–L6 DRGs were rapidly harvested. The Methylated DNA Immunoprecipitation (MeDIP) methods were used to quantify methylation changes in specific DNA regions of *PAX6* and *IRX5*. In short, genomic DNA extracted from DRG was sheared into random fragments using ultrasound instrument Bioruptor and then incubated with monoclonal 5-methylcytosin antibody overnight at 4°C. Next, magnetic beads conjugated to anti-mouse-IgG are used to bind the anti-5mC antibodies, and the precipitate was

rinsed with elution buffer (50 mM Tris-HCl, pH=8.0; 10 mM EDTA, pH=8.0; 1% SDS). DNA was purified with phenol-chloroform solution (phenol to chloroform to isoamyl alcohol=25:24:1) and collected. Finally, qPCR was used to test the expression of PAX6- and IRX5-specific regions. The primer sequences are listed in Table 2.

Chromatin Immunoprecipitation Assay

The kit (ThermoFisher Scientific) was used to perform chromatin immunoprecipitation (ChIP) assays. The rats' L4–L6 DRGs were isolated and placed in 1% formaldehyde for 2 minutes. Sonication was applied to DNA fragments and subsequently digested by micrococcal nuclease. Samples (100 μL) were saved as input after the addition of ChIP dilution buffer. PAX6 antibody or DNMT3b antibody was added to 500 μL for preclearing, and then the samples were incubated overnight. Immunoprecipitation with control mouse IgG (Sigma Aldrich) was performed as a negative control. The DNA was purified from the DNA/antibody complexes through elution and reversion. qPCR was applied on 5 μL of precipitated DNA samples. The primers projected to amplify the specific regions of *TRPC6* were listed in Table 3. Finally, the ChIP to input ratio was calculated.

siRNA Preparation and Screening

Specific siRNAs were applied to knockdown the expression of TRPC6 and PAX6 in a targeted fashion as previously described (Xu et al., 2017). Scramble siRNA without any gene homology was used as a negative control. Briefly, siRNAs designed and synthesized by Ribobio (Guangzhou, China) were firstly transfected into the HBZY-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). TRPC6 and PAX6 expression levels were significantly inhibited by detecting with qPCR (data not shown). Our pilot in vivo experiments showed that intrathecal injection of TRPC6 siRNA or PAX6 siRNA also remarkably suppressed the expression of mRNA and protein expression in DRG of rats (data not shown). The nucleotide sequences of TRPC6 and PAX6 siRNA were shown in Table 4.

Statistical Analysis

All data were expressed as means ± SEM. The results from the behavioral tests, western blot, or qPCR were statistically analyzed with a 1-way or 2-way (repeated-measures) ANOVA followed by Bonferroni post hoc test. The criterion for statistical significance was $P < .05$. Statistical tests were performed with SPSS 25.0 (IBM SPSS).

Table 2. Specific Primer Sequences for MeDIP

Gene	Primer	Sequence
PAX6 (Rat)	Forward	5′- CCAAATCCTGTAACTCATACC -3′
	Reverse	5′- GCTGGACTGAGATTGACTAGG -3′
IRX5 (Rat)	Forward	5′- TTCTATCCGGGCTACACGAAC -3′
	Reverse	5′- TCCGCACGATTCAACACCGTC -3′

Table 3. Specific Primer Sequences for ChIP

Gene	Primer	Sequence
TRPC6 (Rat)	Forward	5′- AACACAGACCGCTCTTCATTTC -3′
	Reverse	5′- AACACTCAGAAAGCAGGGTCC -3′
PAX6 (Rat)	Forward	5′- CCAAATCCTGTAACTCATACC -3′
	Reverse	5′- GCTGGACTGAGATTGACTAGG -3′

Results

Upregulation of TRPC6 in DRG Neurons Contributed to Mechanical Allodynia Induced by Oxaliplatin, Paclitaxel, or Bortezomib Treatment

Consistent with the previous studies (Liu et al., 2016; Xu et al., 2017; Zhang et al., 2018), the mechanical withdrawal threshold of rats was markedly decreased after i.p. administration of oxaliplatin (4 mg/kg once a day for consecutive 5 days), paclitaxel (3×8 mg/kg, once a day on days 1, 4, and 7), or bortezomib (0.2 mg/kg once a day for consecutive 5 days). The mechanical allodynia occurred on day 5 and persisted to day 15 (experiment ended) after chemotherapeutics injection (Figure 1A). The common phenotype and similar time course raised a hypothesis maybe there is a common molecule that mediates mechanical allodynia caused by three different chemotherapeutics in DRG. So we examined the mRNA expression of all ion channels in rats' DRGs by using PCR array. TRPC6 is the only molecular that is significantly upregulated in on 5 and day 15 after all 3 chemotherapeutics treatment (Figure 1B; supplementary Table 1). The results of qPCR and western blot further verified that application of oxaliplatin, paclitaxel, or bortezomib evidently enhanced the mRNA (Figure 1C) and protein (Figure 1D–F) levels of TRPC6 in DRG. Double immunofluorescent staining revealed that TRPC6 was primarily expressed in NF200-positive and IB4-positive neurons but not in GFAP-positive satellite glial cells (Figure 1G–I). Importantly, knockdown of TRPC6 by intrathecal injection of TRPC6 siRNA for 10 consecutive days significantly inhibited the mechanical allodynia induced by oxaliplatin, paclitaxel, or bortezomib (Figure 1J–L). Collectively, these results suggested that TRPC6 may be a common molecular mediating chronic pain induced by different chemotherapeutic agents in DRG.

Chemotherapeutic Treatment Enhanced the DNA Methylation Level of PAX6 Gene

Studies have shown that DNA methylation-mediated epigenetic mechanisms play an important role in neuropathic pain, and DNA methylation is exclusively found in CpG dinucleotides in mammals. To determine whether DNA methylation is involved in the upregulation of TRPC6, we first observed the methylation change in *TRPC6* loci following the oxaliplatin treatment by using RRBS. The results revealed that the methylation level of *TRPC6* loci did not show a difference on day 15 following the oxaliplatin treatment relative to the vehicle group (Figure 2A). These results suggested that TRPC6 upregulation induced by chemotherapeutic drugs did not come from the change of methylation in *TRPC6* loci per se.

Although the methylation level in *TRPC6* loci did not change, 1045 differentially methylated regions (DMRs) on chromosomes were observed following the oxaliplatin treatment (Figure 2B). These DMRs were mapped to 811 genes, and Gene Ontology analysis showed that 80 of these genes were closely related with the nervous system development biological process (Figure 2C;

Table 4. Nucleotide Sequences of TRPC6 and PAX6 siRNA

Gene	Primer	Sequence
TRPC6	Rat	5'- GCUCAGAAGAUUUCCAUUU dTdT -3' (sense)
		5'- AAAUGGAAAUCUUCUGAGC dTdT -3' (antisense)
PAX6	Rat	5'- CUCCAGAAGUUGUAAGCAA dTdT -3' (sense)
		5'- UUGCUUACAACUUCUGGAG dTdT -3' (antisense)

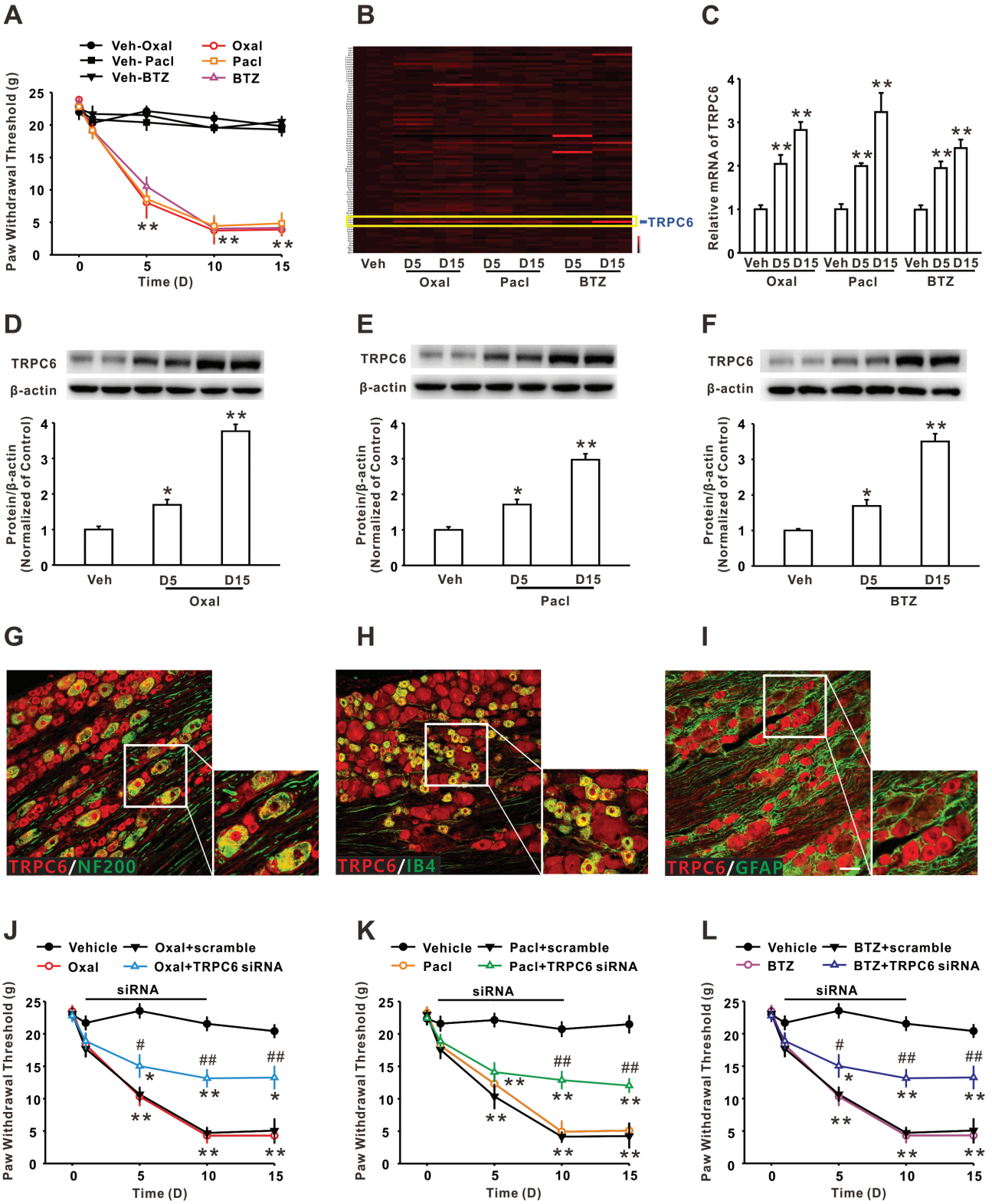

Figure 1. Upregulation of TRPC6 in DRG neurons contributed to mechanical allodynia induced by 3 chemotherapeutics. (A) The hind paw withdraw threshold of rats was obviously reduced following oxaliplatin, paclitaxel, or bortezomib treatment (n=12 in each group; *P<.05, **P<.01 vs the vehicle group). (B) The heatmap of PCR array showed the relative mRNA expression of 84 ion channels in DRG following chemotherapeutics treatment (n=4 in each group). (C–F) The levels of TRPC6 mRNA (C) and protein (D–F) in DRG were significantly upregulated following chemotherapeutics treatment (n=4 in each group; **P<.01 vs the vehicle group). (G–I) The immunofluorescence staining showed the colocalization of TRPC6 (red) with NF200 (a marker for A-type neurons, green) and IB4 (a marker for C-type neurons), but not GFAP (a marker for satellite glial cells, green) in DRG (n=4 in each group), scale bar = 100 μm. (J–L) Intrathecal injection of TRPC6 siRNA for 10 consecutive days obviously relieved the mechanical allodynia in rats treated with oxaliplatin, paclitaxel, or bortezomib (n=12 in each group; *P<.05, **P<.01 vs the vehicle group; #P<.05, ##P<.01 vs the chemotherapeutics group). BTZ, Bortezomib; Oxal, Oxaliplatin; Pacl, Paclitaxel; Veh, Vehicle.

supplementary Table 2). It is well known that transcription factors play an important role in the target protein expression, so we hypothesize that upregulation of transcription factors mediated by the change of methylation may be involved in the increase of TRPC6 following chemotherapeutic drug treatment. By using Genomatix database analysis, we predicted 50 nervous system-related transcription factors closely related to TRPC6 expression (supplementary Table 3). We integrated the analysis of Gene Ontology and Genomatix database and screened 4 transcription factors with potential binding sites to TRPC6 promoter (Figure 2D). Of the 4 transcription factors, PAX6 and iroquois homeobox 5 (IRX5) showed more significant difference of methylation and were chosen for the subsequent study (Figure 2D). Furthermore, we explored the methylation level of DMR in PAX6 or IRX5 by using the anti-5-mc–mediated MeDIP assay. The results showed that the methylation level of *PAX6* DNA, but not *IRX5* DNA, containing the DMR (chr3: 330–440 for *PAX6* and chr19: 4365–4481 for *IRX5*) decreased significantly following oxaliplatin, paclitaxel, or bortezomib treatment (Figure 2E–G). These results implied that the hypomethylation of transcription factor *PAX6* gene may be a common signal event induced by different chemotherapeutic agents.

Chemotherapeutics Treatment Decreased the Recruitment of DNMT3b on PAX6 Gene

It is well known that DNA methyltransferases (DNMT), including DNMT1, DNMT3a, and DNMT3b, via translocating into the nucleus contributed to the DNA methylation in mammals (Ventham et al., 2013; Day et al., 2015; Norollahi et al., 2019). To understand the mechanism underlying the hypomethylation of *PAX6* gene, we first explored the expression of all DNMT mRNA following chemotherapeutics treatment. The results showed that the mRNA level of DNMT3b, but not DNMT1 or DNMT3a, was markedly decreased on day 5 and day 15 following chemotherapeutics injection (Figure 3A–C). Furthermore, the level of DNMT3b protein in nuclei was significantly decreased following the chemotherapeutics treatment compared with the vehicle group (Figure 3D–F). Importantly, ChIP-PCR assay showed that the recruitment of DNMT3b at DMR of *PAX6* gene (+330 – +440) was significantly downregulated after all 3 chemotherapeutics treatments, respectively (Figure 3G–I). These results suggested that chemotherapeutics via decreased levels of nucleus DNMT3b downregulated the recruitment of DNMT3b at the PAX6 gene.

Enhanced Expression of PAX6 in DRG Neurons Involved in Mechanical Allodynia Induced by Chemotherapeutics

Previous study has established that DNA hypomethylation via promoting target gene transcription mediated the target protein upregulation (Ozaki et al., 2017). Here, qPCR and western-blot analysis revealed that chemotherapeutics treatment significantly upregulated the expression of PAX6 mRNA (Figure 4A) and protein (Figure 4B–D) in the DRG on day 5 and day 15. The double immunostaining showed that PAX6 was primarily expressed in NF200-positive and IB4-positive cells, but not GFAP-positive cells (Figure 4E). Importantly, intrathecal injection of PAX6 siRNA for 10 continuous days markedly attenuated the mechanical allodynia induced by oxaliplatin (Figure 4F), paclitaxel (Figure 4G), or bortezomib (Figure 4H) treatment. These results demonstrated that the increased expression of PAX6 in DRG neurons participated in mechanical allodynia induced by chemotherapeutics.

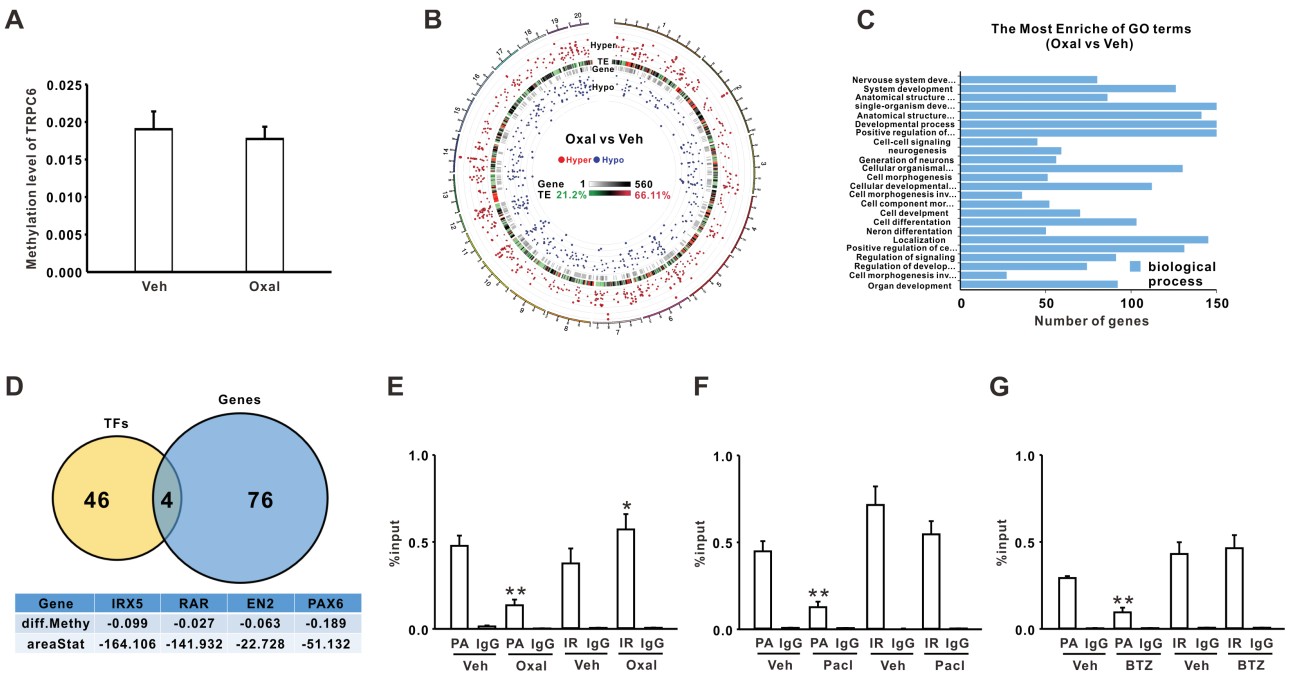

Figure 2. The change of PAX6 gene methylation level followed chemotherapeutics treatment. (A) RRBS results showed the change of methylation level in TRPC6 loci before and after oxliplatin treatment. (B) Circos diagram showed DMRs distributed globally across all chromosomes. (C) The Gene Ontology (GO) pathway analysis showed the enrichment degree of biological process with differential DMR mapped genes. (D) Venn diagram showed the overlap of genes between GO analysis and Genomatix database. (E-G) MeDIP assay was performed to detect the change of DNA methylation level in specific regions of PAX6 and IRX5 loci after chemotherapeutics treatment for 15 days in DRG (n = 3 in each group; *P < .05, **P < .01 vs the correspondence vehicle group). BTZ, Bortezomib; IR, IRX5; Oxal, Oxaliplatin; Pacl, Paclitaxel; PA, PAX6; TFs, Transcription factors; Veh, Vehicle.

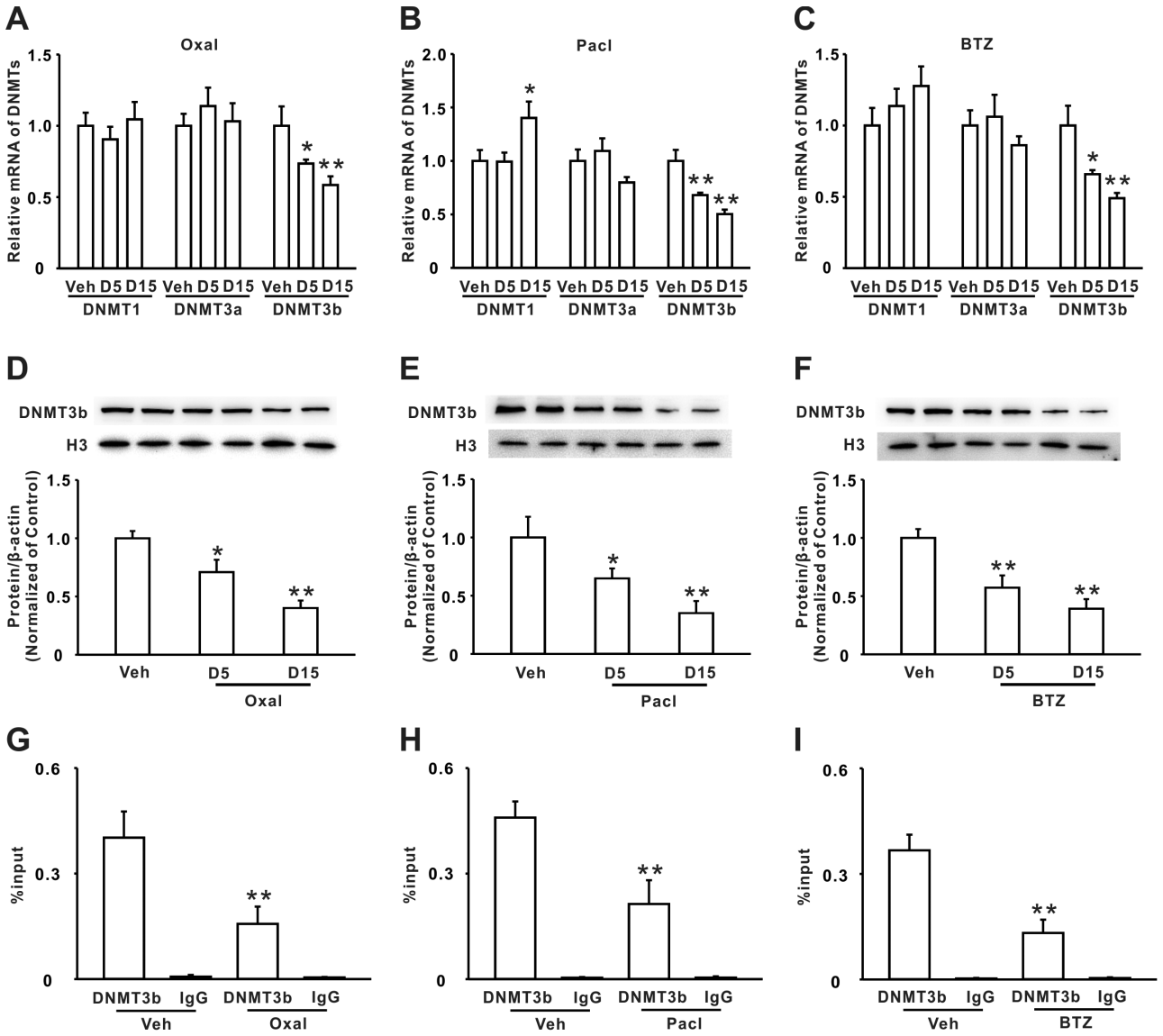

Figure 3. The change of DNMTs in DRG following 3 chemotherapeutics treatment. (A-C) The mRNA levels of DNMT1, DNMT3a, and DNMT3b were explored in DRG on days 5 and 15 following chemotherapeutics treatment (n = 6 in each group; *P < .05, **P < .01 vs the vehicle group). (D–F) Nuclei DNMT3b protein level was significantly decreased following chemotherapeutics treatment on days 5 and 15 (n = 4 in each group; *P < .05, **P < .01 vs the vehicle group). (G–I) ChIP assays were performed with DNMT3b antibody on day 15 following chemotherapeutics treatment (n = 3 in each group; *P < .05, **P < .01 vs the corresponding vehicle group). BTZ, Bortezomib; Oxal, Oxaliplatin; Pacl, Paclitaxel; Veh, Vehicle.

PAX6 Contributed to Upregulation of TRPC6 Following Chemotherapeutics Treatment

Next, we further validated whether the upregulated transcription factor PAX6 mediated the TRPC6 expression following chemotherapeutics treatment. Double immunostaining showed that the expression of PAX6 was colocalized with TRPC6-positive cells in DRG (Figure 5A). Intrathecal injection of PAX6 siRNA for 10 consecutive days obviously prevented the upregulation of TRPC6 protein in DRG on day 15 following chemotherapeutics treatment (Figure 5B–D). Next, TFSEARCH and JASPAR database analysis showed that the position of *TRPC6* (–636/–625) is a potent binding site for PAX6. The DNA, precipitated by the PAX6 antibody, was subjected to PCR to amplify a 128-bp fragment (–727/–600) of the TRPC6 promoter, which contained the PAX6-binding site with the designed primers. ChIP-qPCR analysis revealed that the recruitment of PAX6 to the *TRPC6* promoter was markedly increased on day 15 after application of chemotherapeutics relative to the vehicle group. Importantly, the enhanced binding between PAX6 and *TRPC6* promoter was decreased in rats with application of PAX6 siRNA following chemotherapeutic treatment (Figure 5E–G).

Discussion

It has been suggested that ion channels in DRG neurons are involved in the neuropathic pain following nerve injury or inflammatory (Lin et al., 2016; Mao et al., 2017). TRP channels have a physiological role in the mechanisms controlling several physiological responses like temperature and mechanical sensations (Marwaha et al., 2016). For example, heat activates TRPVs (Guler et al., 2002; Hong and Wiley, 2005), while cold temperatures ranging from affable to painful activate TRPA1 and TRPM8 (McKemy et al., 2002; Story et al., 2003). Among TRPC

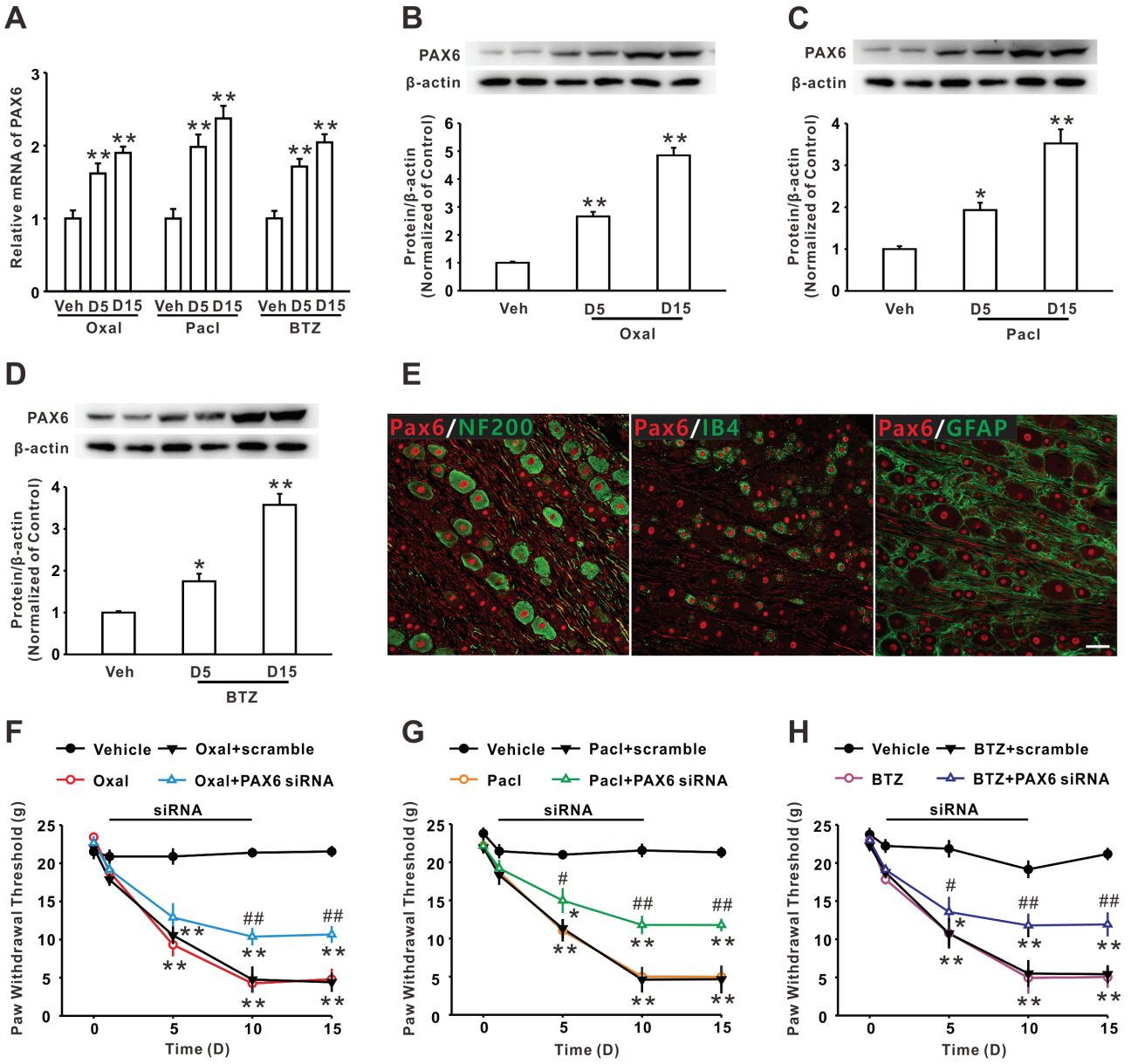

Figure 4. Increased expression of PAX6 in DRG neurons was involved in mechanical allodynia induced by 3 chemotherapeutics. (A) The expression of PAX6 mRNA was significantly increased following oxaliplatin, paclitaxel, or bortezomib treatment (n = 6 in each group; **P < .01 vs the vehicle group). (B–D) Representative blots and histograms showed the total expression of PAX6 protein after chemotherapeutics treatment (n = 4 in each group; *P < .05, **P < .01 vs the vehicle group). (E) The immunofluorescence staining showed the colocalization of PAX6 (red) with NF200 (a marker for A-type neurons, green) and IB4 (a marker for C-type neurons), but not GFAP (a marker for satellite glial cells, green) in DRG (n = 4 in each group), scale bar = 100 μm. (F–H) Intrathecal injection of PAX6 siRNA obviously attenuated the mechanical allodynia induced by oxaliplatin, paclitaxel, or bortezomib (n = 12 in each group; *P < .05, **P < .01 vs the vehicle group, #P < .05, ##P < .01 vs the corresponding chemotherapeutics group). BTZ, Bortezomib; Oxal, Oxaliplatin; Pacl, Paclitaxel; Veh, Vehicle.

channels, a recent study showed that the spinal TRPC6 channel contributes to morphine-induced hyperalgesia (Jin et al., 2017). In the present study, qPCR array analysis first showed that application of 3 chemotherapeutic drugs with different antitumor mechanisms only upregulated the expression of TRPC6 in DRG neurons. Inhibition of TRPC6 by intrathecal injection of TRPC6 siRNA attenuated the mechanical allodynia induced by 3 chemotherapeutics. These results first indicated that TRPC6 upregulation in DRG neurons participated in different chemotherapeutic-induced mechanical allodynia. This result is similar with that TRPC6 is involved in the mechanical hyperalgesia induced by inflammatory or nerve injury (Alessandri-Haber et al., 2009; Roa-Coria et al., 2019). While the possibility existed that the analgesia induced by intrathecal dosing scheme in the present study might be partially supplemented with its potential effect on spinal cord, our results at least showed that the TRPC6 in DRG might be a common therapeutic target for mechanical allodynia induced by chemotherapeutics.

In the current study, we further found that the expression of TRPC6 increased following chemotherapeutic treatment. Given that DNA methylation plays a critical role in the enhancement of target gene transcription, we examined the change of the DAN methylation level of TRPC6. Unexpectedly, the methylation level of TRPC6 loci was not affected following oxaliplatin

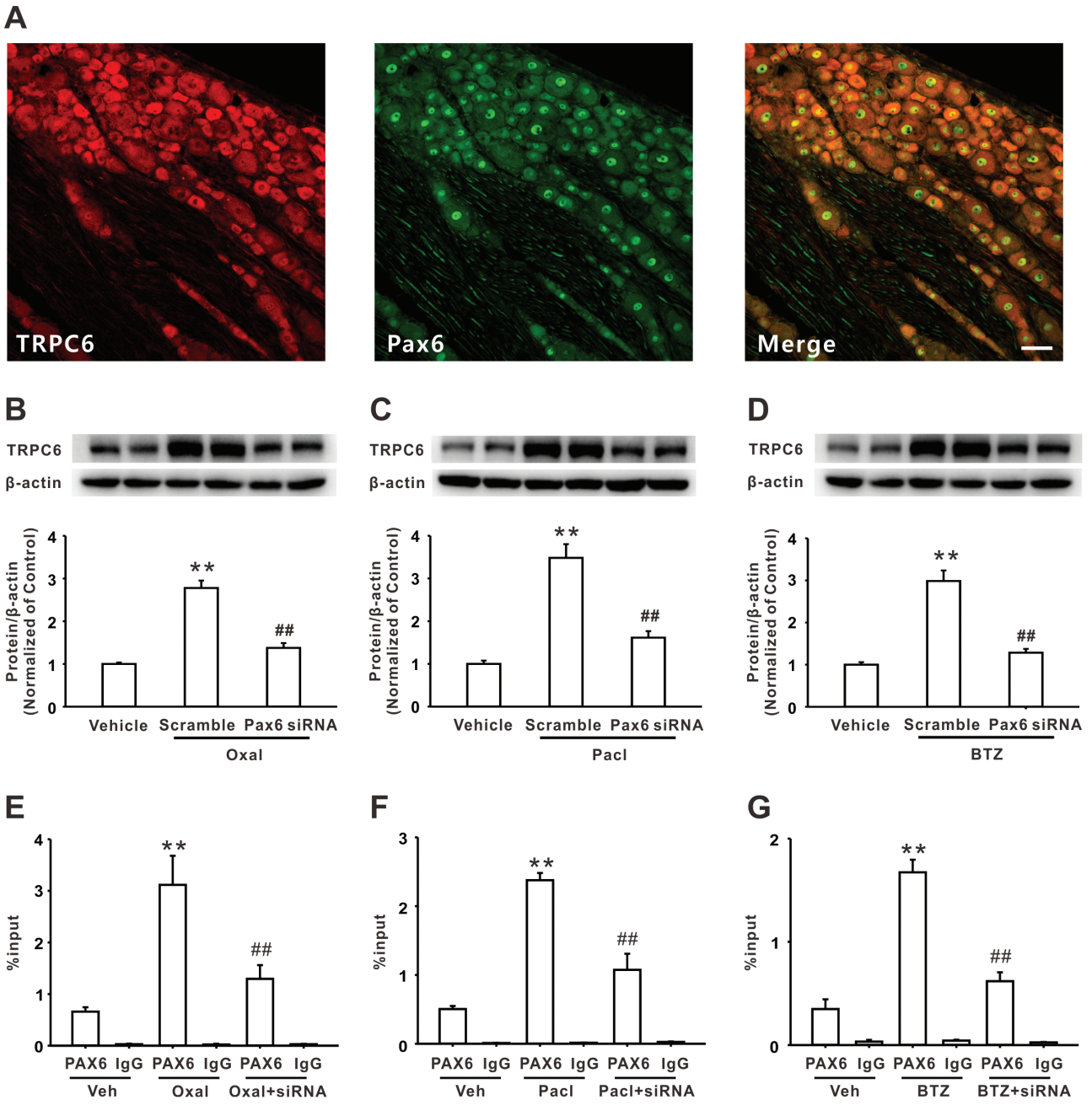

Figure 5. Increased PAX6 mediated TRPC6 upregulation in DRG neurons following chemotherapeutics treatment. (A) The double immunofluorescence staining indicated colocalization of PAX6 with TRPC6 in DRG neurons of rats (n = 4 in each group), scale bar = 100 μm. (B–D) Intrathecal injection of PAX6 siRNA significantly prevented the chemotherapeutics-induced TRPC6 upregulation (n = 4 in each group, **P < .01 vs the vehicle group, ##P < .01 vs the corresponding chemotherapeutics group). (E–G) Following PAX6 siRNA application (i.t.), ChIP assay was performed with PAX6 antibody on day 15 after chemotherapeutics treatment in rats (n = 3 in each group, **P < .01 vs the vehicle group, ##P < .01 vs the corresponding chemotherapeutic group). BTZ, Bortezomib; Oxal, Oxaliplatin; Pacl, Paclitaxel; Veh, Vehicle.

treatment. Studies showed that upregulation and translocation of the transcriptional factor into the nucleus is a canonical way to regulate target gene expression. Furthermore, we found that 80 genes with methylation changes are mainly enriched on the nervous system biological process. Integrated with bioinformatics analysis, PAX6 may be a key transcriptional factor for TRPC6 upregulation, as PAX6 is fit for these criteria that hypomethylation of PAX6 loci and can bind to TRPC6 promotor as a transcription factor. To prove this hypothesis, we first observed the role of PAX6 in the mechanical allodynia. The results showed that chemotherapeutic treatment increased PAX6 expression and local suppression of TRPC6 by using siRNA ameliorated mechanical allodynia. Although evidence showed that PAX6 plays a key role in neurogenesis, which contributed to hyperalgesia in the spinal cord (Rusanescu, 2016), the present study is the first time to testify that PAX6 DRG neurons participated in the chronic pain induced by chemotherapeutics. As a transcriptional factor, PAX6 regulated target gene expression by binding to the promoter (Cvekl et al., 2004). Here, we also found that chemotherapeutics increased the binding between PAX6 and *TPRC6* promoter and application of PAX6 siRNA attenuated the interaction. Moreover, the

increased PAX6 mRNA resulted from the hypomethylation of PAX6 loci mediated by DNMT3b, because the expression of DNMT3b and its binding with PAX6 gene were significantly decreased after application of chemotherapeutics. Up to now, the results first showed that DNMT3b is involved in the hypomethylation of PAX6, suggesting that inhibition of DNMT3b-mediated hypomethylation of PAX6 in DRG might be a novel therapy target for chronic pain.

In conclusion, our study revealed that the activation of PAX6/TRPC6 in DRG neurons may be a common signal pathway for mechanical allodynia following 3 chemotherapeutics application. Furthermore, hypomethylation-mediated epigenetic mechanisms in PAX6 gene might have contributed to the upregulation of the PAX6/TRPC6 pathway. So focusing on PAX6/TRPC6 signaling may provide new ideas and a reliable basis for the treatment of different chemotherapeutics-induced chronic pain.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

Acknowledgments

The authors are entirely responsible for the scientific content of the article.

This study was supported by the National Natural Science Foundation of China (no. 81801103), the China Postdoctoral Science Foundation (nos. 2018M631019 and 2019T120767), the Fundamental Research Funds for the Central Universities (No. 18ykpy41), the Guangzhou Innovation Platform construction Project (No. 2060404), the Natural Science Foundation of Guangdong Province (2019A1515010871), and the 111 Project (No. B13037).

Statement of Interest

None.

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