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Histone H3K9 methylation regulates chronic stress and IL-6-induced colon epithelial permeability and visceral pain

Running title: Histone regulation of GI permeability and pain

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Abstract

Background

Chronic stress is associated with activation of the HPA-axis, elevation in pro-inflammatory cytokines, decrease in intestinal epithelial cell tight junction (TJ) proteins and enhanced visceral pain. It is unknown whether epigenetic regulatory pathways play a role in chronic stress-induced intestinal barrier dysfunction and visceral hyperalgesia.

Methods

Young-adult male rats were subjected to water avoidance stress ± H3K9 methylation inhibitors or siRNAs. Visceral pain response was assessed. Differentiated Caco-2/BBE cells and human colonoids were treated with cortisol or IL-6 ± antagonists. Expression of TJ, IL-6 and H3K9 methylation status at gene promoters were measured. Transepithelial electrical resistance and FITC-Dextran permeability were evaluated.

Key Results

Chronic stress induced IL-6 upregulation prior to a decrease in TJ proteins in the rat colon. The IL-6 level inversely correlated with occludin expression. Treatment with IL-6 decreased occludin and induced visceral hyperalgesia. Chronic stress and IL-6 increased H3K9 methylation and decreased transcriptional GR binding to the occludin gene promoter, leading to down-regulation of protein expression and increase in paracellular permeability. Intra-rectal administration of a H3K9 methylation antagonist prevented chronic stress-induced visceral hyperalgesia in the rat. In a human colonoid model, cortisol decreased occludin expression which was prevented by the GR antagonist RU486, and IL-6 increased H3K9 methylation and decreased TJ protein levels which were prevented by inhibitors of H3K9 methylation.

Conclusions & Inferences

Our findings support a novel role for methylation of the repressive histone H3K9 to regulate chronic stress, pro-inflammatory cytokine-mediated reduction in colon TJ protein levels, increase in paracellular permeability and visceral hyperalgesia.

Keywords: chronic stress, visceral hyperalgesia, epithelial cell tight junctions, histone methylation, pro-inflammatory cytokines

Key points

- IL-6 levels increase prior to down-regulation in tight junction proteins in colon epithelial cells. IL-6 decreased tight junction protein levels, increased paracellular permeability and induced visceral hyperalgesia.
- Chronic stress and IL-6 increased H3K9 methylation at tight junction gene promoters. H3K9 methylation antagonists decreased chronic stress and IL-6 induced down-regulation in tight junction proteins and paracellular permeability.
- Our findings support a novel role of H3K9 methylation in regulation of intestinal epithelial paracellular permeability and visceral pain.

Introduction

In humans with Irritable Bowel Syndrome (IBS) and validated rodent models, chronic stress is associated with enhanced visceral pain (visceral hyperalgesia),[1-3] low-grade inflammation that involves recruitment of immune cells and elevated levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and TNF-α.[4-8] The increase in cytokine levels in IBS patients correlates with increased intestinal epithelial cell paracellular permeability that is associated with down-regulation in intestinal epithelial tight junction (TJ) proteins,[9-11] including claudin(s) and occludin.[12] The increase in permeability correlates with visceral hyperalgesia in patients with IBS-D, individuals exposed to combat conditions and chronic stress rodent models.[13-17] It is unknown if chronic stress-associated increase in pro-inflammatory cytokines precedes or is a manifestation of intestinal barrier

dysfunction. The cellular and molecular pathways that regulate chronic stress-induced down-regulation in epithelial cell tight junction gene expression are poorly understood.

Chronic stress-associated visceral hyperalgesia involves inducible and potentially reversible changes in gene expression which implicates epigenetic regulatory pathways in the process. Epigenetics is commonly defined as heritable alterations in gene expression that do not arise from altered DNA sequence, usually involving environmentally-induced changes in chromatin such as DNA methylation, histone modification, and nucleosome positioning.[18-20] Interest in the role of epigenetics and gene function has emerged rapidly because of its significance in numerous important physiological and pathophysiological processes.[21-23] Evidence supports a potentially pivotal role for epigenetic pathways in the regulation of pain signaling, including chronic stress-associated visceral hyperalgesia in validated rodent models.[23-25] We reported previously that chronic stress is associated with region-specific alterations in pain signaling in dorsal root ganglion neurons innervating the rat colon[26] but not sciatic nerve distribution, and decreased intestinal epithelial TJ protein expression that inversely correlated with the magnitude of enhanced visceral pain.[15,27] It is largely unknown whether epigenetic pathways play a role in regulating epithelial tight junction gene expression and function in chronic stress-associated visceral hyperalgesia.

Histones are functionally characterized by small chemical modifications that alter their interaction with DNA. The “histone hypothesis” stipulates that certain combinations of histone modifications may function, alone or together, as a part of a predictive histone code to provide ground rules for chromatin folding and, thereby, gene availability for transcription. For example, methylation of lysine 9 on histone 3 (H3K9me2/me3), catalyzed by methyltransferases such as G9a (Ehmt2), GLP (Ehmt1) and Suv39h1/Suv39h2, is usually associated with silenced gene transcription and condensed chromatin.[28,29] On the other hand, methylation of lysine 4 on histone 3 (H3K4me3) is strongly enriched at promoter regions of active genes where chromatin exists in an open conformation.[30,31] In this study we focused on the potential role of H3K9 methylation in chronic stress and IL-6 mediated down-regulation in colon epithelial cell TJs because of its well described role in gene repression.

In summary, the goal of this body of work was to explore a potentially novel role for the repressive histone H3K9 to regulate chronic stress-induced, pro-inflammatory cytokine-mediated down-regulation in colon epithelial cell TJ proteins, increase in paracellular

permeability and visceral hyperalgesia. We identified a predominant role for histone H3K9me2 at promoters of occludin and claudin-1 genes, performed *in vivo* studies to examine the effect of selective antagonists that prevent the repression of H3K9me2 at occludin/claudin(s) gene promoters, paracellular permeability and visceral hyperalgesia in the chronic intermittent water avoidance stress rat model. Complementary translational studies were performed using differentiated human Caco-2/BBE cells and human colonoids.

Materials and Methods

Animals and water avoidance (WA) stress

The animal study was conducted blindly as described previously.[15,32] Young-adult male Sprague-Dawley rats (weighting 160-180 g) were obtained from Charles River Laboratories (Wilmington, MA) and subjected to water avoidance (WA) stress for 10 consecutive days. The animals received a standard laboratory diet and tap water ad libitum and were adapted in the animal facility for 5-7 days before experimental procedures. All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to National Institutes of Health guidelines.

Measurement of the visceral motor response (VMR) to colorectal distention (CRD) was conducted in awake animals as described previously.[25,32] The VMR was quantified by measuring activity of electromyography (EMG) in the external oblique musculature in the awake animals. CRD was conducted to constant pressures of 20, 40, 60 and 80 mmHg by a custom-made distension control device. The increase in the area under curve (AUC), which is the sum of all recorded data points multiplied by the sample interval (in seconds) after baseline subtraction, was presented as the overall response during the course of the CRD test.

Treatment in rats in situ

In some rat groups, the in-vivo stable H3K9 methylation antagonist UNC0642 (1 mg/kg body weight; Cayman Chemical, Ann Arbor, MI), a potent and preferential inhibitor of histone methyltransferases G9a and GLP which catalyze H3K9me and H3K9me2,[33-35] or DMSO vehicle was delivered to the rat distal colon region (1-5 cm to anal opening) and incubate for 2 hours every two days during the 10-day stress period. Several other groups

of rats were administrated similarly to the distal colon region with 10-25 ng IL-6 (in 1 ml delivery media; R&D Systems, Minneapolis, MN) based on the previous report,[36] siRNA (10 nM) or vehicles and incubated for 2 hours using the method as we described previously.[15] Pre-designed occludin-specific siRNA (Catalog# 4390816) and negative control siRNA (Catalog# 4390844) were obtained from Thermo Fisher Scientific (Austin, TX). In some animals, GR antagonist RU486 (2 mg/kg body weight; Cayman Chemical) was administrated subcutaneously to the stressed rats daily during 10-day stress phase as we described previously.[25,27] Visceral motor response (VMR) to colorectal distention (CRD) were conducted on the next day after completing the 10-day stress procedure or 48 hours after IL-6 or siRNA treatment.

Cell culture and treatment

The Caco-2/BBE (American Type Culture Collection, Manassas, VA) epithelial cells were maintained at 37°C in 10% CO_2. Cells underwent differentiation for 21 days with medium changes every three days. Cells were then incubated with cortisol (500 nM; Cayman Chemical) or IL-6 (10 ng/ml) for 24 hours ± the H3K9 methylation inhibitor UNC0638 (500 nM; Cayman Chemical) that inhibits G9a and GLP catalyzing H3K9me and H3K9me2[35,37] and BRD4770 (10 μM; Cayman Chemical) that inhibits G9a and selectively reduces H3K9me2 and H3K9me3. DMSO at the final 0.01% (v/v) concentration was used as the vehicle control.

Measurement of transepithelial electrical resistance (TEER) and FITC-Dextran permeability

The TEER assay was performed as described previously.[38] Caco-2/BBE cells were cultured on 24-well 12 mm polyester Transwells (Corning, Corning, NY) for 21 days. IL-6 (10 ng/ml) was added to both the upper and lower chambers of the transwell cultures. TEER was measured before and after treatment for 24 hours ± UNC0638 (500 nM) or BRD4770 (10 μM) or vehicle using an EVOM2 epithelial voltage meter (World Precision Instruments, Sarasota, FL). TEER values were calculated after subtraction of the intrinsic resistance of the cell-free filter. To measure dextran permeability, 4 kDa fluoroscein isothiocyanate (FITC)-dextran (FD4; 3 mg/ml) was added to the upper chamber without medium change. Aliquots were withdrawn from the lower chambers after 4 h and assayed for fluorescence at 515 nm with excitation at 492 nm.

Human colonoid culture

The protocol for colon biopsy collections from patients was approved by the Institutional Review Board of the University of Michigan Hospital with written informed consent. Isolation of healthy human male colonic crypts and culture of colonoids were performed using the previously described protocol.[39,40] Briefly, human colon biopsies were collected and incubated with 10 mM DTT (Sigma-Aldrich, St Louis, MO) for 15 min and then with 8 mM EDTA for 75 min at 4°C. The crypts were isolated in KGMG media (Lonza, Walkersville, MD) with Matrigel (BD Biosciences, San Jose, CA). Crypts were then dissociated and incubated in L-WRN medium (10% fetal bovine serum), supplemented with 500 nM A83-01 (Tocris), 10 μM SB202190 (Sigma), and 10 μM Y27632 (Tocris). For the first 10 days of culture, the medium was also supplemented with 2.5 μM CHIR99021 (Tocris). For the experimental phase, established colonoids were interrogated in a mix of L-WRN culture medium diluted 1:4 with KGM Gold medium with 2.5% final serum and 0.25 mM calcium. Organoid cultures were passaged every 4-7 days by digesting Matrigel in cold 2 mM EDTA and plated on the first day with 10 μM Y27632 (Miltenyi Biotec). Colonoids were treated with cortisol (5 μM) or IL-6 (10 ng/ml) ± RU486 (500 nM) or UNC0638 (500 nM) for 24 hours.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously.[25] DNA was sonicated to random fragments between 200 bp to 500 bp. The chromatin was subjected to immunoprecipitation using the following antibodies: GR (# 3660; Cell Signaling Technology, Danvers, MA, US) and H3K9me2 (# A-4035; Epigentek, Farmingdale, NY, USA). Primers for rat occludin promoter (forward- GCTGGAACAAGCATTTGGATAC, reverse- GCCAGGAGGCTGATAAGAAC), claudin-1 promoter (forward- AGGCAACCAGAGCCTTGATGGTAA, reverse- CATGCACTTCATGCCAATGGTGGA) and for human occludin promoter (forward- CGACACACCACACCTACACT, reverse- ATGCGCACCAACGTGGAA) were obtained from Life Technologies (Grand Island, NY, US). PCR was performed using Bio-Rad iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA, US).

Quantitative PCR

Total RNA was isolated from Caco-2/BBE cells and human colonoids using the RNeasy kit (Qiagen). Q-PCR was performed using Bio-Rad iScript™ One-Step RT-PCR Kit with SYBR® Green using the following specific primers from IDT (Integrated DNA

Technologies, Coralville, IA): occludin (forward- GGAAGGTTCTGGTGTGAACTAA; reverse- CTGAAAGGTGGTTGAGAGGATTA), claudin-1 forward- CCAGTTAGAAGAGGTAGTGTGAAT, reverse- CAGCCAGCTGAGCAAATAAAG), claudin-4 (forward- CCTCGTCATCATCAGCATCAT, reverse- CTTTCATCCTCCAGGCAGTT), IL-6 (forward- GGAGACTTGCCTGGTGAAA, reverse- CTGGCTTGTTCCTCACTACTC), GR (forward- GGGAAACGCTCTGACATACA, reverse- CTTCTGTGGGTACGTGTCTTAG), GAPDH (forward-AAGGTGAAGGTCGGAGTCAA, reverse-AATGAAGGGGTCATTGATGG). Gene expression (mRNA) data were normalized to GAPDH.

Western blot and immunofluorescence

Immunoblot was conducted as described previously.[25,38] Colon tissues from rats were dissected out next day after completing the 10-day WA stress procedure or 48 hours after IL-6 or siRNA treatment in control rats. Then tissues were incubated with 4 mM EDTA in cold PBS for 5 min with shaking. The epithelium layers were gently scrapped off and colon crypts were collected. Proteins were extracted using the lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1.5 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and Complete Protease Inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). In separate studies, human Caco-2/BBE cells and colonoids were collected and homogenized using the same lysis buffer. Proteins were separated and blotted with following primary antibodies: GR (Cell Signaling, Danvers, MA), IL-6 (Millipore Sigma, St. Louis, MO), claudin-1 (Invitrogen Corp., Camarillo, CA), occludin (Abcam, Cambridge, MA), occludin (Cell Signaling), claudin-4 (Thermal Fisher Scientific), β-actin (Sigma-Aldrich). The immunoblot bands were scanned and quantitated using NIH ImageJ software. The expression levels of proteins of interest were normalized to β-actin for comparison.

For immunofluorescence staining, colonoids were isolated from Matrigel using 2 mM EDTA and fixed in 10% formalin for 1 hour. Colonoids were then permeabilized and blocked with 10% normal goat serum in PBS with 0.3% Triton X-100 for 4 hours at room temperature. Primary antibodies used for overnight incubation were anti-occludin (Cell Signaling), Ki67 (Abcam, Cambridge, MA, USA) and CK20 (US Biological, Swampscott, MA, USA). Secondary antibodies Alexa Fluor 488 (1:500) and Alexa Fluor 594 (1:500) from Molecular Probes (Life Technologies) were used for incubation for 2 hours. The

slides were mounted with Prolong Gold containing DAPI nuclear stain (P36935; Life Technologies Molecular Probes).

Statistics

To assess the VMR in response to CRD pressures, the EMG amplitudes, represented by calculating the area under curve (AUC), were normalized as percentage of baseline response for the highest pressure for each rat and then averaged for each group of rats. The effects of stress and/or IL-6 treatment on the VMR to CRD was analyzed using a repeated-measures two-way ANOVA followed by Bonferroni post-test comparisons. Unpaired Student t test was used to examine the data for protein, mRNA, TEER, and ChIP assays. Results were expressed as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Chronic stress induced elevation in IL-6 prior to the down-regulation in occludin and GR receptor

To examine the relationship between chronic stress, pro-inflammatory cytokine IL-6, and epithelial TJ proteins, we performed a time-course study to measure the protein levels in rat colon epithelial cells in chronically stressed male rats. As shown in Fig. 1, IL-6 was significantly increased on day 3 during the 10-day WA stress procedure compared with the controls and displayed the same trend on day 6 and day 11 ($P < 0.05$; $n = 5$). The TJ protein occludin was significantly decreased on day 6 and day 11 ($P < 0.05$) whereas it showed modest change on day 3 during the stress phase (Fig. 1B). Furthermore, the expression of GR receptor demonstrated a trend of down-regulation during the stress phase and was significantly decreased on day 6 and day 11 in the colon epithelial cells in the stressed rats ($P < 0.05$; Fig. 1C). In addition, we analyzed the relationship between expressions of IL-6 and occludin in the colon in the same animal in the control and 10-day stressed rats. As depicted in Fig. 1D, protein levels of IL-6 and occludin varied among the control and stressed rats, but revealed an inverse relationship that a higher level of IL-6 expression correlated with a lower level of occludin in the same animal. A significant linear negative correlation was observed with a Pearson coefficient of -0.789 ($P < 0.001$) between occludin and IL-6 (Fig. 1D).

IL-6 decreased occludin expression and induced visceral hyperalgesia in the rat

To examine the effect of IL-6 on occludin gene and protein expression, two doses of IL-6 (10 ng/ml and 25 ng/ml) were administrated into the distal colon in separate healthy control rat groups. As shown in Fig. 2A, occludin mRNA level in colon epithelial cells was significantly decreased 24 hours after treatment with IL-6 at both doses of 10 ng/ml and 25 ng/ml compared with vehicle controls ($P < 0.05$; $n = 4$). Moreover, administration of IL-6 to the distal colon induced significant decrease in occludin protein expression in the rat colon at both doses (Fig. 2B&C). Moreover, VMR to CRD was significantly increased 84.8 ± 15.2% and 70.3 ± 21.5% at the distention pressure of 40 mm Hg and 60 mm Hg, respectively, in animals treated with IL-6 (10 ng/ml) compared with vehicle controls (Fig. 2D). No significant difference in VMR to CRD was observed between untreated rats and vehicle treated rats. To examine the hypothesis that decreased occludin expression could result in visceral hyperalgesia, healthy control rats were treated with occludin specific siRNA delivered intra-rectally. As shown in Fig. 2E, occludin siRNA treated rats displayed significant decrease in occludin protein level compared with those rats treated with scrambled, negative siRNA ($P < 0.05$, $n = 5$). In addition, silencing of occludin in the rat colon induced an increase in visceral pain perception (Fig. 2F). EMG activity increased to 85.8 ± 10.9%, 128.7 ± 7.6%, and 166.7 ± 7.2% at the distention pressure of 20 mm Hg ($P < 0.001$), 40 mm Hg ($P < 0.01$) and 60 mm Hg ($P < 0.001$; $n = 5$), respectively, in rats treated with occludin siRNA, while VMR was 16.2 ± 3.1%, 72.7 ± 7.6% and 100.0 ± 12.9% in rats treated with vehicle, respectively, at the distention pressure of 20 mm Hg, 40 mm Hg and 60 mm Hg. No significant difference in VMR to CRD was observed between scrambled, negative siRNA treated rats and vehicle treated rats.

Chronic stress induced alterations of H3K9 methylation at tight junction gene promoters in the rat colon epithelial cells

It has been reported that methylation of H3K9 is associated with heterochromatin and repressed (limited) gene transcription.[41,42] In chronically stressed rats, distinct alterations of H3K9 methylation were observed in colon epithelial cells. As shown in Fig. 3A&B, both H3K9me2 and H3K9me3 were significantly increased in the chronically stressed rats compared with controls. H3K9me2 increased 101.5 ± 25.1% ($P < 0.01$; $n = 4$) and H3K9me3 increased 168.3 ± 47.5% ($P < 0.05$; $n = 4$) in stressed rats compared with controls, while H3K9me was not significantly changed ($P = 0.109$). ChIP analysis revealed that H3K9me2 level was significantly increased at gene promoters of occludin (129.5 ±

71.7%) and claudin-1 (98.7 ± 29.8%) in stressed rats ($P < 0.05$; $n = 3$) compared with controls as shown in Fig. 3C. In addition, binding of GR, an activating transcription factor for both occludin and claudin-1 genes, was significantly decreased at promoter regions of occludin (35.8 ± 15.2%) and claudin-1 (32.3 ± 8.2%) genes in the colon crypts of stressed rats ($P< 0.01$; $n = 3$), which was prevented by GR antagonist RU486 (Fig. 3D&E).

Inhibition of H3K9 methylation prevented chronic stress-induced decreases in tight junction proteins and visceral hyperalgesia

To evaluate the effect of H3K9 methylation in vivo, stressed rats were treated with UNC0642 (a more stable inhibitor of H3K9 methylation employed in in-vivo experiments compared to UNC0638 which is commonly used in in-vitro experiments), a potent and preferential inhibitor of histone methyltransferases G9a and GLP which catalyze H3K9me and H3K9me2.[33-35] Both occludin and claudin-1 proteins were significantly decreased in stressed rats treated with vehicle (for UNC0642) compared with controls rats treated with vehicle ($P < 0.05$; $n = 5$), which is consistent with our previous observation that these TJ proteins decreased in chronic stress.[27] Furthermore, when stressed animals were treated with the selective H3K9me2 inhibitor UNC0642 during the stress phase, the down-regulation of occludin and claudin-1 expression in colon epithelial cells was restored to the control levels (Fig. 4A&B). As shown in Fig. 4C, chronic stress induced significant increases in VMR in response to CRD at the distention pressures of 40, 60, and 80 mmHg in rats with vehicle treatment ($P < 0.05$; $n = 5$). UNC0642 treatment during the stress phase significantly decreased VMR to CRD at pressures of 60 and 80 mmHg compared with stressed rats treated vehicle ($P < 0.05$; $n = 5$). In stressed rats treated with vehicle, the EMG activities were 188.9 ± 9.9% and 209.5 ± 18.0% at the distention pressures of 60 mmHg and 80 mmHg, respectively. The EMG activities were decreased to 103.3 ± 26.7% and 123.0 ± 26.0% at the distention pressures of 60 mmHg and 80 mmHg, respectively, in stressed rats treated with UNC0642. No significant differences were observed in EMG activity at distention pressures of 20 mmHg and 40 mmHg between the stressed rats with UNC0642 and stressed rats treated with vehicle.

Cortisol and IL-6 induced increases in H3K9me2 and paracellular permeability in Caco-2/BBE cells

We reported previously that cortisol treatment decreased occludin and claudin-1 expression and increased paracellular permeability in differentiated human Caco-2/BBE

cells.[38] To examine the potential involvement of H3K9 methylation in this process, differentiated Caco-2/BBE cells were treated with cortisol and IL-6 in the presence/absence of a potent H3K9 methylation antagonist UNC0638, which inhibits G9a and GLP. As shown in Fig 5A, cortisol treatment significantly increased IL-6 mRNA expression (125.6 ± 46.8%) compared to the control ($P < 0.05$; $n = 6$). ChIP analysis revealed that the H3K9me2 level was significantly increased at both occludin and GR gene promoters after cortisol treatment ($P < 0.05$; $n = 5$) as shown in Fig. 5B. In Caco-2/BBE cells treated with IL-6, the protein levels of occludin and claudin-1 were significantly decreased, which was prevented by co-treatment with the selective H3K9 methylation inhibitor UNC0638 (n=5, Fig. 5C&D). In addition, paracellular permeability, assessed with TEER, was significantly decreased in differentiated Caco-2/BBE cells cultured in transwells ($P < 0.01$; $n = 8$). The decrease in TEER observed after IL-6 treatment was prevented by the selective G9a and GLP inhibitor UNC0638 ($P < 0.05$, $n = 8$), whereas a selective G9a inhibitor BRD4770[43,44] showed a modest effect on TEER (Fig. 5E). As shown in Fig. 5F, FD4 (4 kDa FITC-dextran) paracellular permeability was significantly increased 53.2 ± 25.0% compared to the controls after IL-6 treatment ($P < 0.05$; $n = 8$), which was decreased significantly to 85.1 ± 10.8% of the controls in the presence of UNC0638 ($P < 0.01$). Treatment with BRD4770 also significantly decreased FD4 permeability to 116.8 ± 3.3% of the control ($P < 0.05$; $n = 8$).

Cortisol treatment decreased occludin expression in differentiated human colonoids

To further examine the translatability of the rodent and cell studies to the human, we employed an *in vitro* human colonoid model to test whether cortisol plays a role in regulating intestinal epithelial TJ and barrier function in primary human colonoid preparations. As shown in Figure 6A, tissue-derived human colonoids cultured in differentiation media for 48 hours, displayed crypt-like budding robustly expressing the marker of differentiation cytokeratin 20 (CK20), expression of occludin and poorly expressed the stem cell proliferating marker Ki67 (Fig. 6A). These data indicate that differentiated tissue-derived human colonoids are a viable new model that reproduces observations in human biopsies and animal models. In the differentiated human colonoids, cortisol treatment significantly decreased occludin expression as shown in Fig. 6B. Level of occludin mRNA decreased to 68.6 ± 3.2% of the control after cortisol treatment ($P < 0.01$; $n = 6$), which was prevented by co-treatment with GR antagonist RU486 ($P < 0.01$).

Claudin-1 mRNA was also significantly decreased to 75.8 ± 4.7% of the control after cortisol treatment ($P < 0.01$; $n = 6$) which was prevented in the presence of RU486 ($P < 0.05$). In contrast, claudin-4 mRNA level was not altered after treatment with cortisol alone or treatment with cortisol and RU486. In addition, cortisol treatment induced a 121.5 ± 33.2% increase in IL-6 mRNA level in human colonoids ($P < 0.01$; $n = 5$) while co-treatment with RU486 modestly reduced the mRNA level of IL-6. On the other hand, cortisol treatment significantly decreased mRNA expression of GR ($P < 0.05$; $n = 5$). Co-treatment with RU486 had no significant effect on the expression of GR mRNA in human colonoids (Figure 6C).

IL-6 altered promoter H3K9me2 level and protein expression of occludin and claudin-1 in human colonoids

ChIP analysis revealed increases of H3K9me2 levels at occludin and claudin-1 promoter regions after IL-6 treatment in differentiated human colonoid preparations (Fig. 7A&B). H3K9me2 levels at occludin and claudin-1 gene promoters were increased to 142.4 ± 12.4% and 171.4 ± 57.9% in human colonoids after IL-6 treatment ($P < 0.05$; $n = 4$), respectively. The preferential H3K9 methylation inhibitor UNC0638 restored H3K9me2 level to 78.6 ± 3.2% and 92.8 ± 11.8% of controls at occludin and claudin-1 gene promoters in human colonoids ($P < 0.05$; $n = 4$), respectively. Consistently, mRNA level of occludin decreased to 72.8 ± 9.2% in human colonoids after IL-6 treatment ($P < 0.05$; $n = 5$), which was prevented by UNC0638 co-treatment. Claudin-1 mRNA level was decreased to 65.7 ± 10.3% of the control after IL-6 treatment ($P < 0.05$; $n = 5$), while UNC0638 had modest restoration of claudin-1 mRNA (Fig. 7C)

Discussion

Enhanced abdominal pain (visceral hyperalgesia) is commonly observed in patients with IBS and chronic stress animal models.[1-3] Intestinal barrier dysfunction appears to be a significant contributing factor to chronic stress-associated visceral hyperalgesia.[15,27,45] Chronic stress is associated with elevation in intestinal mucosa pro-inflammatory cytokines levels including IL-1β, IL-6, IL-8 and TNF-α,[4,8,46] and can also decrease the expression of specific TJ proteins directly via reduced levels of GR receptor that serves

as a positive transcription factor at promoter sites of several epithelial TJ genes.[47] We focused on the role of IL-6 because literature and screening RNA-seq studies using colon mucosa specimens obtained from chronic stress rats revealed that elevation in the level of IL-6 was particularly robust, consistent with the literature.[48] It is unknown whether elevation in pro-inflammatory cytokines precedes or is a consequence of intestinal barrier dysfunction. In time course studies we observed that IL-6 was significantly increased early during the WA stress phase compared with the control and displayed the same trend during the late stress phase. In contrast, the TJ protein occludin was significantly decreased during the late phase of WA stress procedure. Concurrent profiling in protein expression of IL-6 and occludin in colon epithelial cells revealed an inverse relationship. These data suggest that elevation in pro-inflammatory cytokine IL-6 may act as a contributing factor leading to down-regulation in TJ proteins. This was supported by the fact that IL-6 treatment significantly decreased occludin expression in the rat *in vivo* and Caco-2/BBE cells *in vitro*. Occludin is a structural and regulatory protein that modulates epithelial barrier function together with paracellular pore-forming protein claudins.[49] Decreased expression in TJ proteins could disrupt TJ pores resulting in increased permeability to macromolecules and ions, which is linked to a variety of local and systemic diseases.[49-52] The observation that application of IL-6 increased paracellular permeability further confirmed the linkage of pro-inflammatory cytokines and intestinal barrier dysfunction. It will be important to compare our results obtained with IL-6 with other relevant pro-inflammatory cytokines in future studies.

Recent research supports a potentially pivotal role for epigenetic regulatory pathways in chronic stress-associated visceral hyperalgesia because these pathways play an important role in inducible and reversible gene expression, including receptors involved in pain signal transduction such as the anti-nociceptive endocannabinoid-1 receptor and pro-nociceptive receptor TRPV1.[25,53,54] Epigenetics has been defined as “the study of changes in gene function that are mitotically and/or meiotically heritable that do not entail a change in DNA sequence”.[55] In practice, epigenetic modifications comprise histone variants, post-translational modifications of amino acids on the amino-terminal tail of histones, and covalent modifications of DNA bases. It is broadly accepted that epigenetic modifications include a role for non-coding RNAs which can play an important role in environmentally-induced changes in chromatin accessibility.[56] We observed that IL-6 decreased GR transcriptional binding to the occludin gene promoter that was associated with increases in repressive H3K9me2 at the occludin gene promoter, decrease in occludin protein

expression and increase in paracellular permeability. The decrease in TEER and increase in FD4 permeability induced by IL-6 were prevented by specific H3K9 methylation inhibitors, suggesting H3K9 methylation plays an important role in regulation of TJ protein expression and epithelial paracellular permeability. It is likely that DNA methylation at TJ gene promoters will also be altered by chronic stress or IL-6, which links to H3K9 methylation to repress gene expression.

Previous studies have demonstrated a strong correlation between reduced epithelial TJ protein expression, barrier dysfunction and visceral hyperalgesia.[27,57-59] Occludin siRNA–treated control rats demonstrated a significant increase in VMR to CRD. This finding was reproduced in the chronic stress rat model that displayed distinct alterations in H3K9 methylation at occludin and claudin-1 gene promoters in colon epithelial cells. It is likely that increases of H3K9 methylation at gene promoters altered chromatin structure and blocked transcription factor binding leading to gene repression. This interpretation was supported by *in vivo* experiments using an *in vivo* stable, selective H3K9 methylation inhibitor UNC0642 to treat animals during the chronic stress phase. UNC0642 treatment significantly decreased visceral hyperalgesia and prevented the down-regulation in occludin and claudin-1 protein expression in colon epithelial cells in stressed rats. The preventative effect of UNC0642 on chronic stress-induced reduction of TJ protein occludin and claudin-1 is likely to alleviate the increase in epithelial paracellular permeability and subsequently prevents visceral hyperalgesia in stressed rats. It is important to note that our observations support a role for both H3K9 dimethylation and H3K9 trimethylation in the regulation of colon epithelial cell TJ gene expression, although H3K9 dimethylation may have a predominant role under our experimental conditions. Future studies will be need to determine whether there are specific roles for H3K9 dimethylation and trimethylation involving specific TJ proteins. Furthermore, this study employed the validated male WAS model to examine the role of H3K9 methylation due to less variability when compared to studies using female rats, presumably because of the confounding effects of the estrus cycle on pain perception. However, this does not negate the essential need for comparative studies using female rats, particularly, in view of the clinical observation that functional pain and IBS are more prevalent in females.[60,61] It is noteworthy that IBS is commonly observed in military personnel exposed to combat conditions, a predominately male population.[16,17]

To examine whether the observations in the rodent are translatable to the human we performed complementary studies using differentiated human colonoids. These experiments demonstrated that: i) Cortisol treatment significantly decreased TJ protein occludin and claudin-1 levels in human colonoids that was prevented by co-treatment with the GR antagonist RU486. It's noteworthy that cortisol treatment had no significant effect on mRNA expression of claudin-4 while co-treatment with GR antagonist RU486 induced a modest increase in claudin-4 mRNA in human colonoids, suggesting that RU486 influences claudin-4 expression. Claudin-4 can either act as a conventional TJ barrier or a cation-barrier depending on the cell type and the species.[12,62] The effects of CORT and RU486 are dose- and duration-dependent, and influenced by the animal and cell models employed which can have distinctive expression profiles for GR. The mechanism(s) underlying the increased expression of claudin-4 by RU486 in colonoids requires more detailed experiments in future studies; ii) Cortisol treatment induced a significantly increase in IL-6 mRNA in human colonoids while it decreased GR mRNA expression. Cortisol-induced down-regulation of the GR receptor supports a feedback control mechanism. The relationship between activation of GR and expression of IL-6 and the question of whether an increase in IL-6 can inhibit GR expression requires further comprehensive analysis; and iii) Increases in H3K9me2 levels at TJ claudin-1 and occludin gene promoter regions after IL-6 treatment in human colonoid preparations were prevented by the H3K9me2 inhibitor UNC0638. IL-6 treatment significantly decreased occludin mRNA levels in human colonoids which was prevented by UNC0638. Taken together, these results support that the observations in the chronic stress rat model are translatable to the human.

While our results support a potentially important role for H3K9me2 in chronic stress and IL-6 induced decrease in epithelial TJ protein expression, increase in paracellular permeability and visceral hyperalgesia, other pathways likely play a role. We chose to focus on H3K9 methylation because of its known role as a repressive histone associated with reduced gene expression.[63] However, methylation of other repressive histones such as H3K27 may play a role in regulation of epithelial barrier function.[64] It is also possible that reciprocal changes (decreased function) in activator histones such as H3K4 that are associated with active gene expression may also play a role in down-regulation in intestinal epithelial TJ gene and protein expression (ibid). Patients with diarrhea-prone IBS and a mouse model of chemically-induced colitis demonstrated increased expression of microRNA 29 and reduced levels of claudin-1 and nuclear factor–kB-repressing factor,

increased intestinal permeability and visceral hyperalgesia.[65,66] Chronic stress-associated visceral hyperalgesia is also influenced by biological sex. For example, epidemiology studies indicate that IBS demonstrates an increased prevalence in females in the general population,[67] although the diagnosis is common in combat veterans (a largely male population).[68] It will be important to compare our results in male rats with female rats, as well as, human males and females with IBS in future studies. Research also supports a central (CNS) role for epigenetic mechanisms in visceral hyperalgesia under chronic, intermittent stress conditions.[69-71] It is unclear whether the reported changes in the CNS require peripheral input and/or occur independently. Other studies examining the effects of histone deacetylase (HDAC) inhibitors indicate that epigenetic pathways modulate pain perception in mouse models of neuropathic and inflammatory pain.[72-75]

In summary, we believe that this is the first report that provides a compelling body of evidence supporting a potentially significant role for a specific histone pathway involving H3K9me2/me3 in the regulation of chronic stress-induced visceral hyperalgesia via IL-6 induced, GR-mediated down-regulation of intestinal epithelial TJ gene transcription and expression (occludin and claudin-1) in intestinal epithelial cells, increased paracellular permeability, and enhanced visceral pain sensation (summarized in Figure 8). These observations are potentially relevant to patients with IBS who frequently report stress-rated, enhanced abdominal pain. It is noteworthy that chronic stress- and pro-inflammatory cytokine-mediated increase in intestinal epithelial permeability has been implicated in the pathophysiology of numerous medical conditions including inflammatory bowel disease, Celiac disease, graft vs host disease and type 1 diabetes mellitus,[51,76] suggesting that the proposed role for H3K9 methylation may have impact beyond visceral hyperalgesia. With the emergence of molecularly targeted, region-specific interventions in the GI tract, these observations have the potential to lead to novel treatments for disorders associated with enhanced intestinal epithelial cell paracellular permeability.[77]

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Disclosures:

The authors have declared that no conflict of interest exists.

Author Contribution:

John W Wiley and Shuangsong Hong planned, designed the study and wrote the article; Ye Zong, Gen Zheng and Shengtao Zhu conducted experiments; Ye Zong and Shuangsong Hong analyzed the data, and all authors contributed to review of the manuscript.

Abbreviations: ChIP, Chromatin Immunoprecipitation; CRD, colorectal distention; GR, glucocorticoid receptor, H3K9me2, histone 3 lysine 9 dimethylation; IBS, Irritable Bowel Syndrome; TEER, transepithelial electrical resistance; VMR, visceral motor response; WA, water avoidance

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Figure Legends

Figure 1: Time course study of differential expression of IL-6, occludin, and GR receptor in the rat colon during 10-day water avoidance (WA) stress procedure. (A) Alterations of relative amount of IL-6 in the control and stressed rats (n = 4 for each group) during the 10-day stress phase. (B) Changes in the relative amount of tight junction occludin in the control and stressed rats (n = 4 for each group) during the 10-day stress phase. (C) Alterations of relative amount of GR receptor in the control and stressed rats (n = 4 for each group) during the 10-day stress phase. (D). Inverse correlation of the protein expression level between IL-6 and occludin in the colon of same individual control and stressed (after 10-day stress) rats (n = 6 for each group). Relative expressions of IL-6, occludin, and GR were obtained from intensities of immunoblot bands from the same rat sample and normalized to β-actin. Data were expressed as mean ± SEM. *, $P < 0.05$.

Figure 2. IL-6 treatment induced down-regulation of occludin in the colon of healthy control rats *in vivo*. (A) Changes in the mRNA expression of occludin in the colon epithelial cells in control rats treated with IL-6 (10 ng/ml and 25 ng/ml) in the distal colon. (B) Treatment of IL-6 at the dose of 10 ng/ml, indicated as IL-6 (10), and 25 ng/ml, indicated as IL-6 (25), in the distal colon of control rats decreased occludin protein expression compared with vehicle controls. (C) Bar graph showing the significant changes in occludin protein expression in the colon in control rats treated with IL-6 (10 ng/ml and 25 ng/ml) or vehicles. (D) Increased VMR to CRD in healthy rats treated with IL-6 (10 ng/ml) in the distal colon compared with untreated or vehicle-treated controls. (E) Occludin siRNA treatment decreased occludin expression in the rat colon compared to the scrambled, negative siRNA-treated. (F) Silencing of occludin using siRNA delivered to the distal colon increased visceral motor response to colorectal distention in the healthy rats. The changes in protein expression were normalized to β-actin. n = 4-5. *, $P < 0.05$.

Figure 3. Changes in H3K9 methylation status at tight junction gene promoters in chronically stressed rats. (A) Immunoblotting of H3K9me, H3K9me2 and H3K9me3 in colon epithelial cells in healthy control and WA stressed rats. (B) Significant increases in H3K9me2 and H3K9me3 levels were observed in the rat colon in stressed rats compared with controls. (C) ChIP analysis demonstrated increases in H3K9me2 at occludin and

claudin-1 gene promoter in stressed rats. (D) GR binding to claudin-1 and occludin gene promoters in the colon epithelial cells in control, stressed rats ± GR antagonist RU486. ChIP neg: ChIP with normal rabbit IgG; ChIP pos: ChIP using input DNA prepared from control rat colon epithelial cells; input: 4% input DNA from relevant epithelial cells for normalization; PCR neg: no input DNA. (E) Significant decreases in GR binding to occludin and claudin-1 gene promoters were observed in stressed rats, which was prevented by GR antagonist RU486. n = 3-4. *, $P < 0.05$; **, $P < 0.001$.

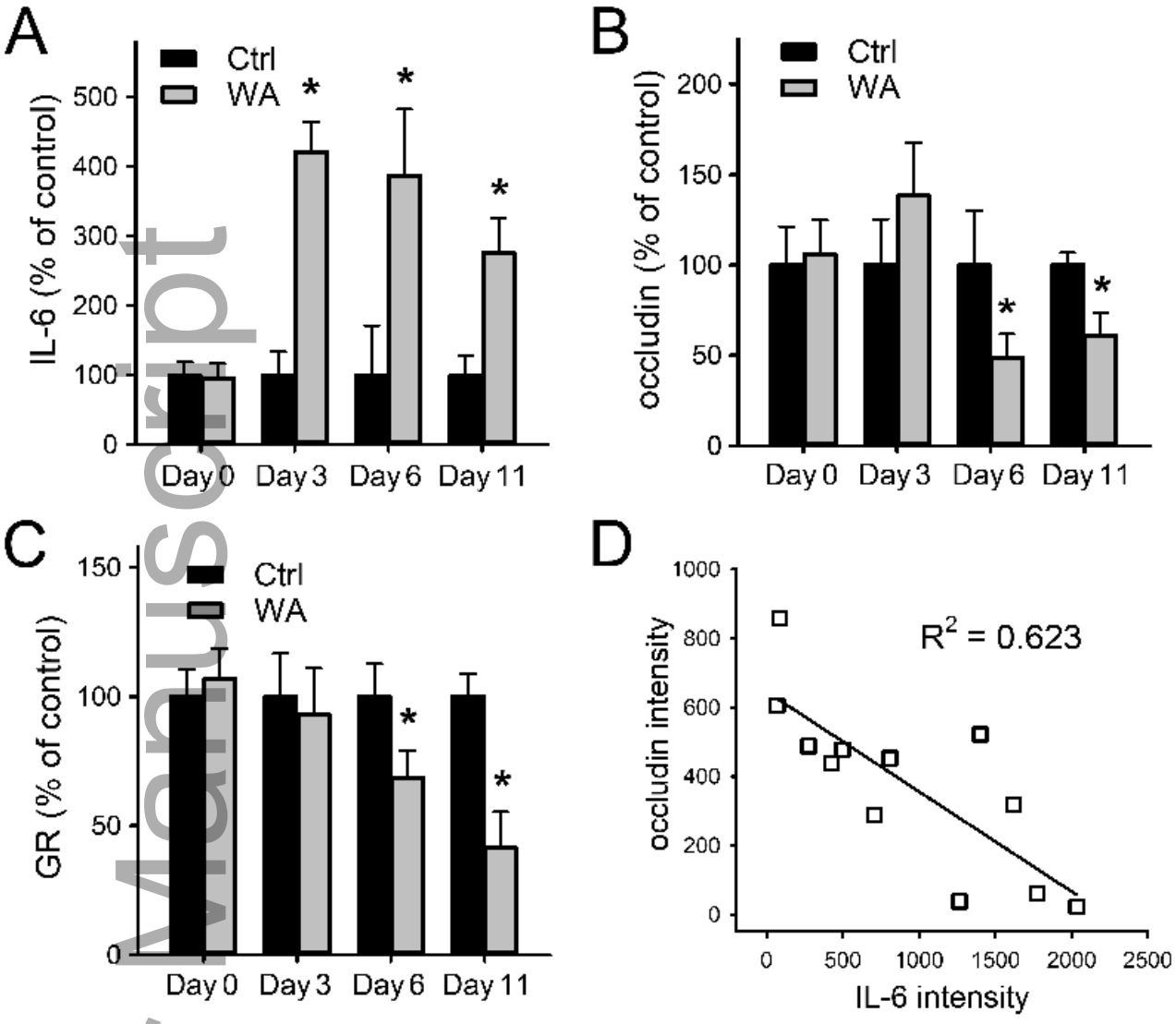
Figure 4. Treatment with the stable, preferential H3K9 methylation antagonist UNC0642 blocked chronic stress-induced down-regulation of occludin and claudin-1 in the rat colon and prevented visceral hyperalgesia. (A) Immunoblots of occludin and claudin-1 in the colon epithelial cells in stressed rats treated with UNC0642, control rats treated with vehicle, and stressed rats treated with vehicle. (B) Statistical analysis of occludin and claudin-1 protein levels in the colon in stressed rats ± UNC0642. (C) UNC0642 treatment prevented chronic stress-induced increase in visceral pain perception in the rat. n = 5; *, $P < 0.05$ for stressed rats compared with control rats; #, $P < 0.05$ for stressed rats compared to UNC0642-treated stressed rats.

Figure 5. Changes in H3K9 methylation and paracellular permeability in differentiated Caco-2/BBE cells after cortisol and IL-6 treatment. (A) mRNA expression of IL-6 in Caco-2/BBE cells after cortisol treatment (1 μM, 24 hours). (B) Increased H3K9me2 levels at occludin and GR promoters in Caco-2/BBE cells after cortisol treatment (1 μM, 24 hours). (C) Immunoblots for occludin and claudin-1 in cells treated with IL-6 (10 ng/ml) for 24 hours with/without preferential H3K9 methylation antagonist UNC0638 (500 nM). (D) Statistical graph showing significant decreases in occludin and claudin-1 in cells treated with IL-6, which were prevented by UNC0638. (E) TEER measurement in Caco-2/BBE cells treated by IL-6 (10 ng/ml) for 24 hours with/without H3K9me2 antagonist UNC0638 (500 nM) or BRD4770 (10 μM). (F) Changes in FD4 (4 kDa FITC-dextran) permeability in Caco-2/BBE cells after IL-6 (10 ng/ml) treatment for 24 hours with/without UNC0638 (500 nM) or BRD4770 (10 μM). n = 5-8; *, $P < 0.05$; **, $P < 0.01$.

Figure 6. Cortisol induced down-regulation in occludin and claudin-1 in differentiated human colonoids. (A) Morphology of human colonoids cultured in differentiation media for 0 h (a) and 48 h (b). Confocal microscopy demonstrated low levels of the stem cell proliferating Ki67 (green, panel c) and robust expression of the marker of cell differentiation CK20 (red, panel c) and occludin (green, panel d) in colonoids after differentiation for 48 hours. DAPI was used for counter staining. Scale bar: 50 μm. (B) Real-time PCR analysis for occludin and claudin-1 mRNAs in differentiated human colonoids after cortisol (CORT) treatment (5 μM) for 24 hours ± GR antagonist RU486 (500 nM). RU486 prevented CORT-induced decreases in occludin and claudin-1 and increased the expression of claudin-4. (C) Changes in mRNA expression of IL-6 and GR in human colonoids treated with CORT (5 μM) for 24 hours ± RU486 (500 nM). n = 6. *, $P < 0.05$; **, $P < 0.01$.

Figure 7. The H3K9 methylation antagonist UNC0638 prevented IL-6 induced decreases in occludin and claudin-1 expression in differentiated human colonoids. (A) ChIP analysis using H3K9me2 antibody of occludin promoter region after IL-6 (10 ng/ml) treatment ± UNC0638 (500 nM) for 24 hours. (B) ChIP by H3K9me2 of claudin-1 promoter region after IL-6 (10 ng/ml) treatment ± UNC0638 (500 nM) for 24 hours. (C) Quantitative PCR analysis of claudin-1 and occludin in human colonoids treated with IL-6 (10 ng/ml) for 24 hours ± UNC0638 (500 nM). n = 4 - 5. *, $P < 0.05$; **, $P < 0.01$.

Figure 8. Proposed model for the role of the repressive histone H3K9 methylation on pro-inflammatory cytokine (IL-6)-mediated, down-regulation of intestinal epithelial cell TJ gene transcription, protein expression and intestinal barrier dysfunction in chronic stress-associated visceral hyperalgesia. Chronic stress increases levels of pro-inflammatory cytokines such as IL-6 that promotes methylation of repressive H3K9me2 and H3K9me3 in the colon epithelial cells. This leads to decreased binding of transcription factors, such as GR, at epithelia cell tight junction gene promoters, culminating in reduced TJ gene transcription and protein expression. The down-regulation of TJ proteins, including occludin, claudin-1 and ZOs, results in increased paracellular permeability and enhanced visceral pain sensation.



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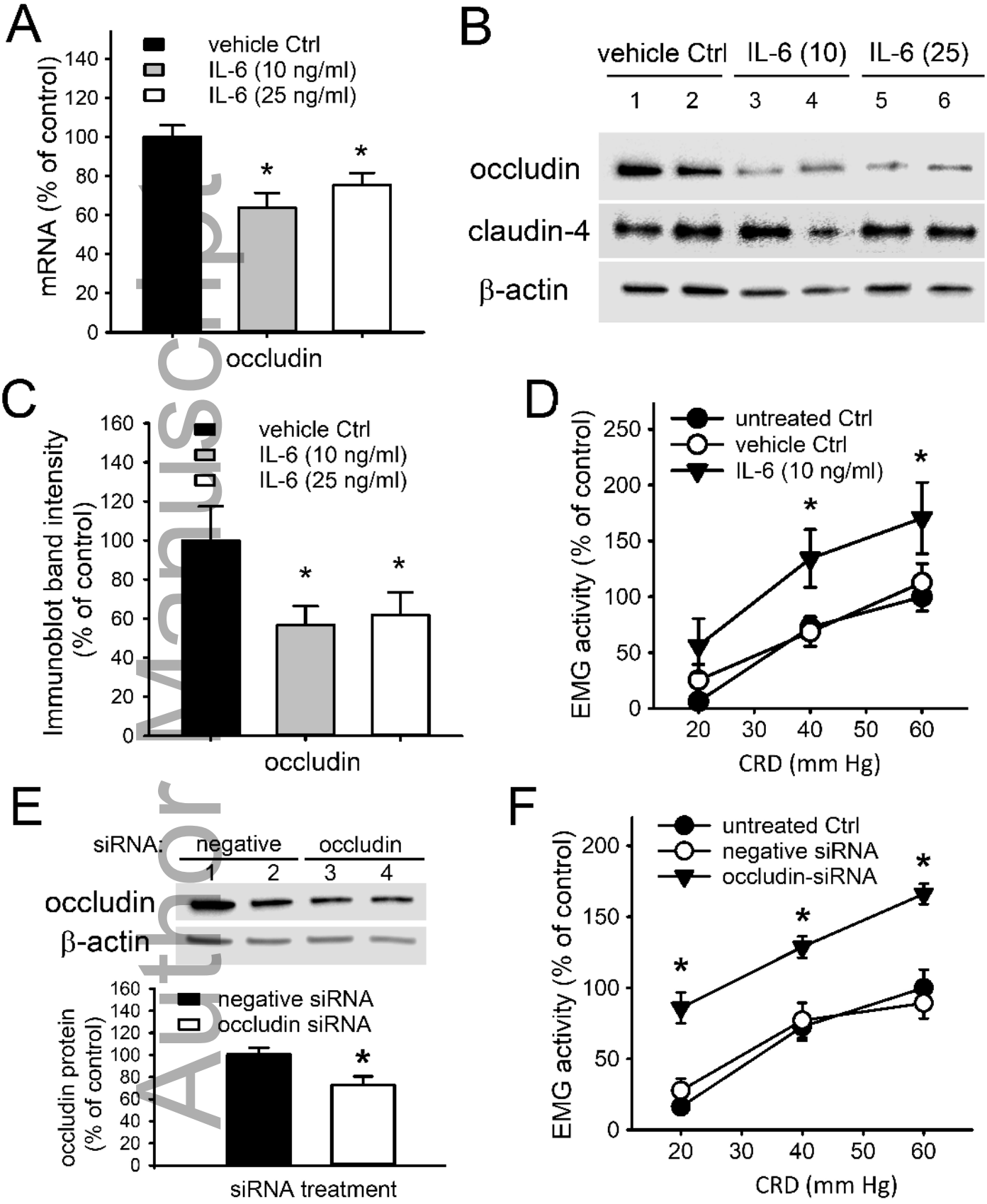

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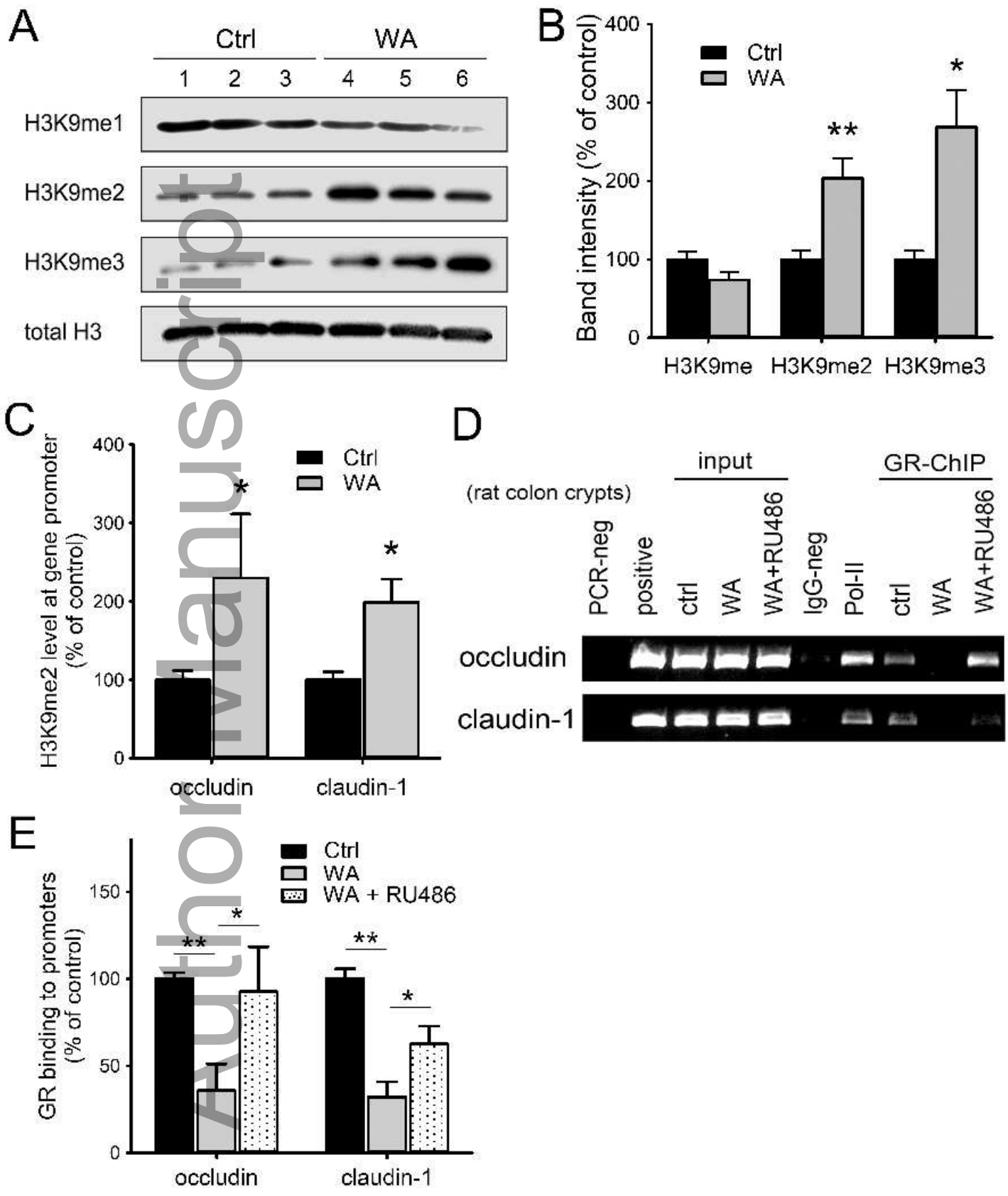

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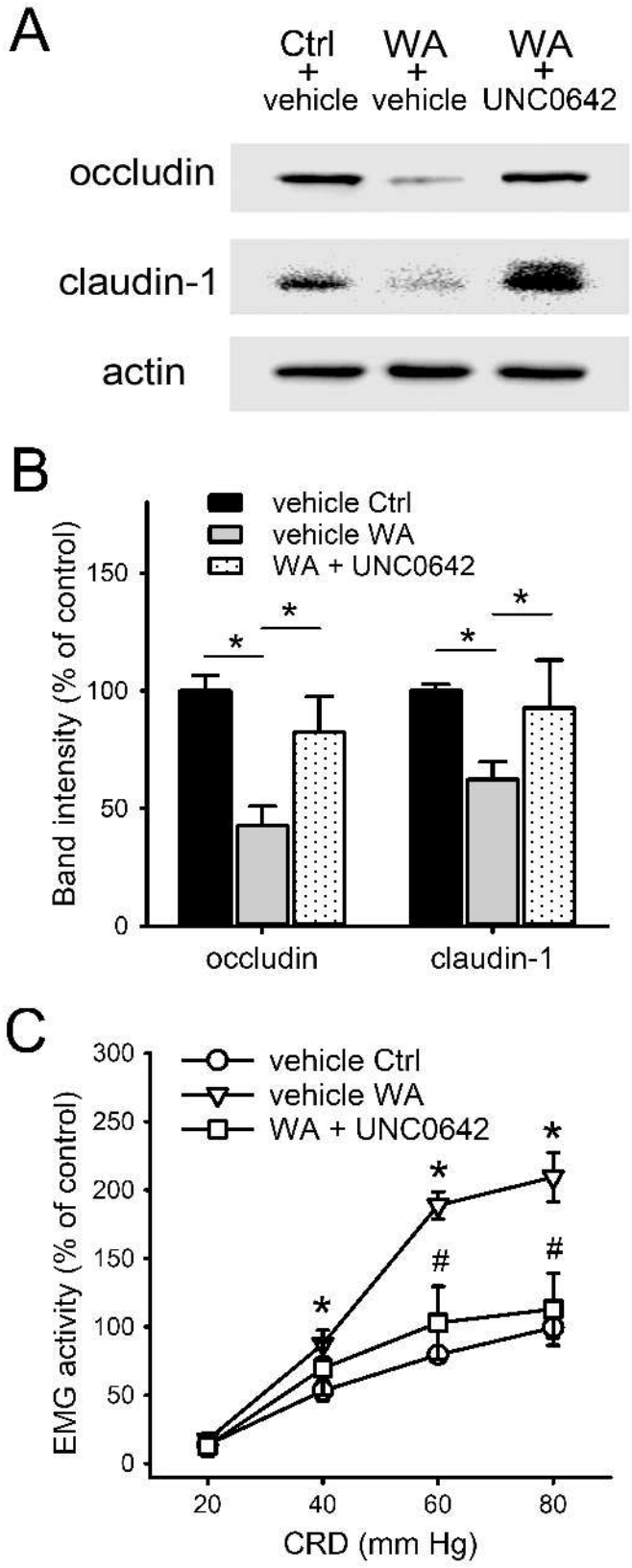

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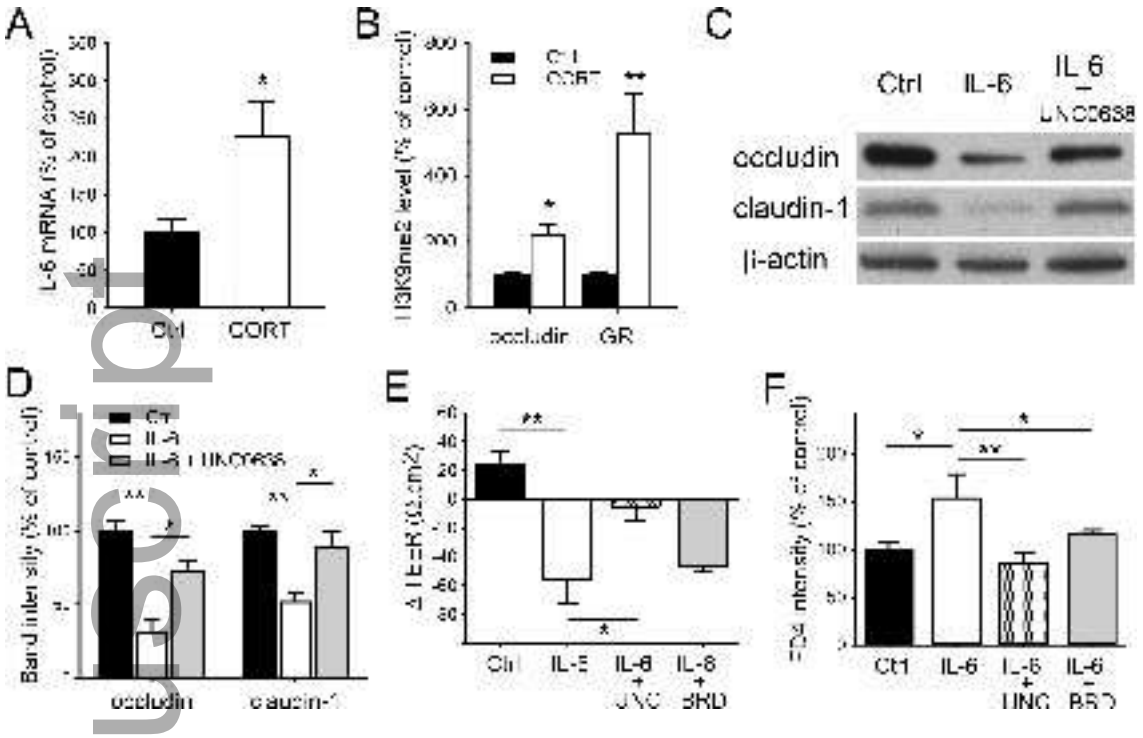

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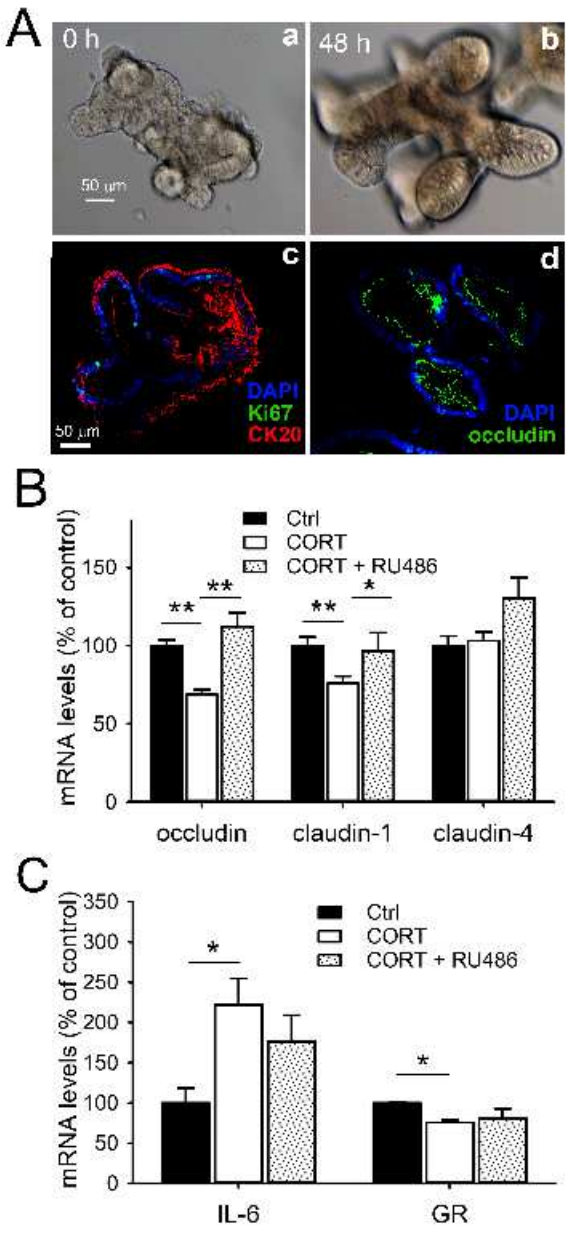

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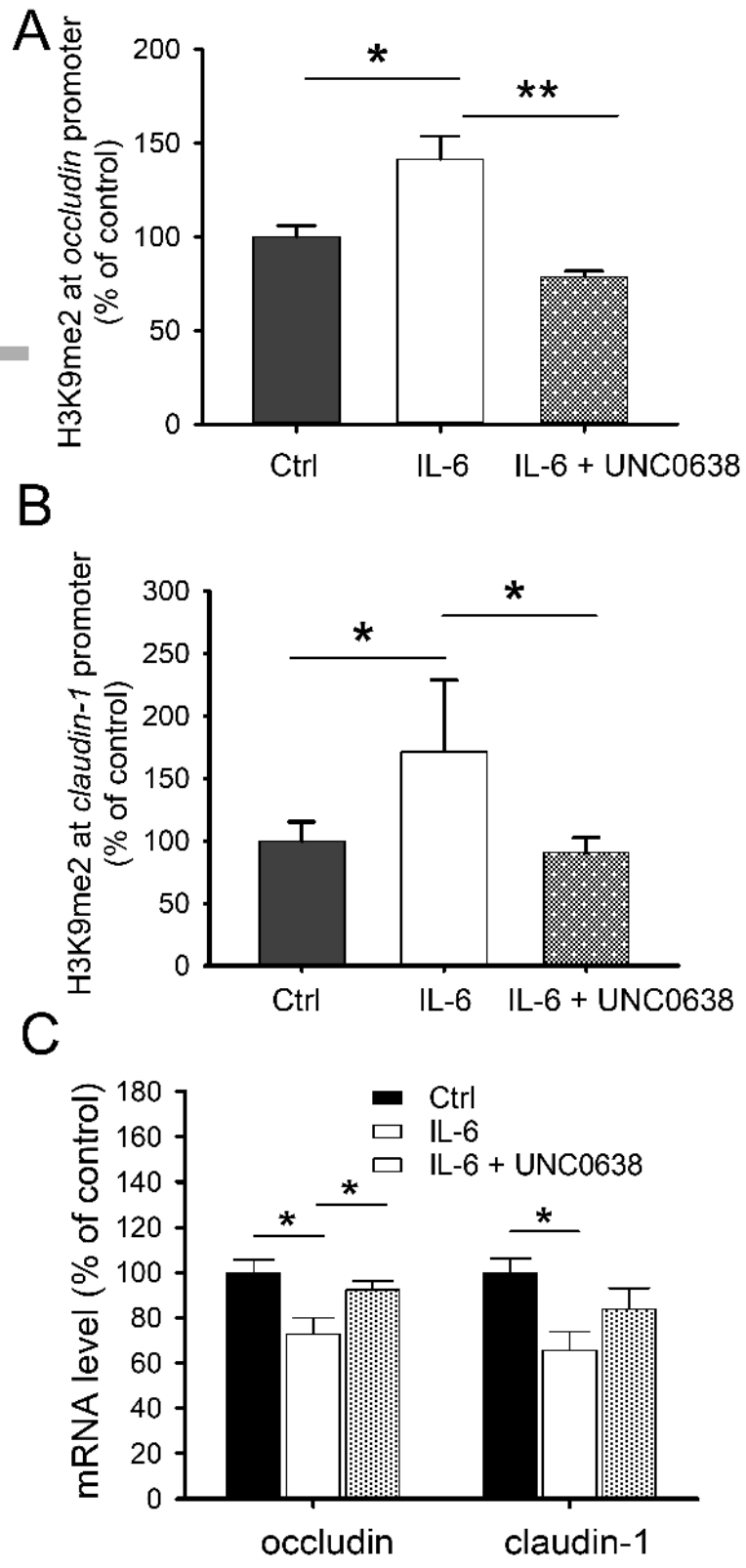

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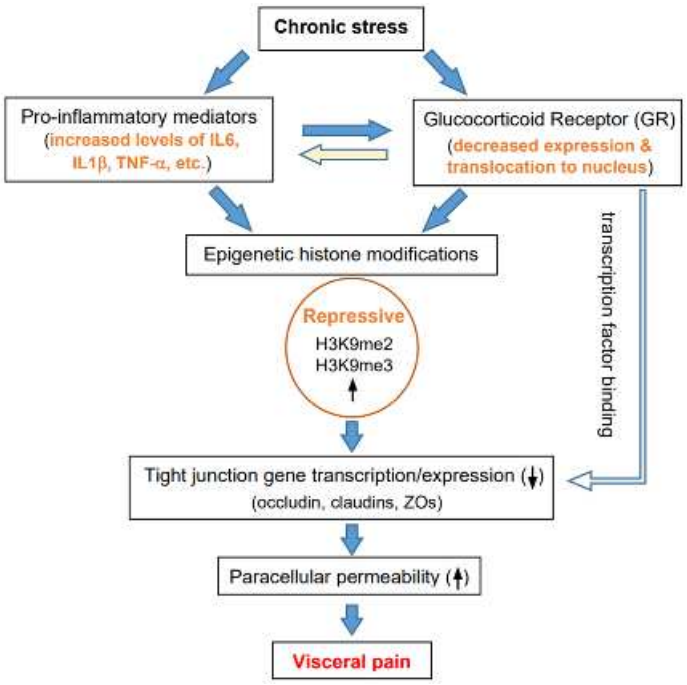

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