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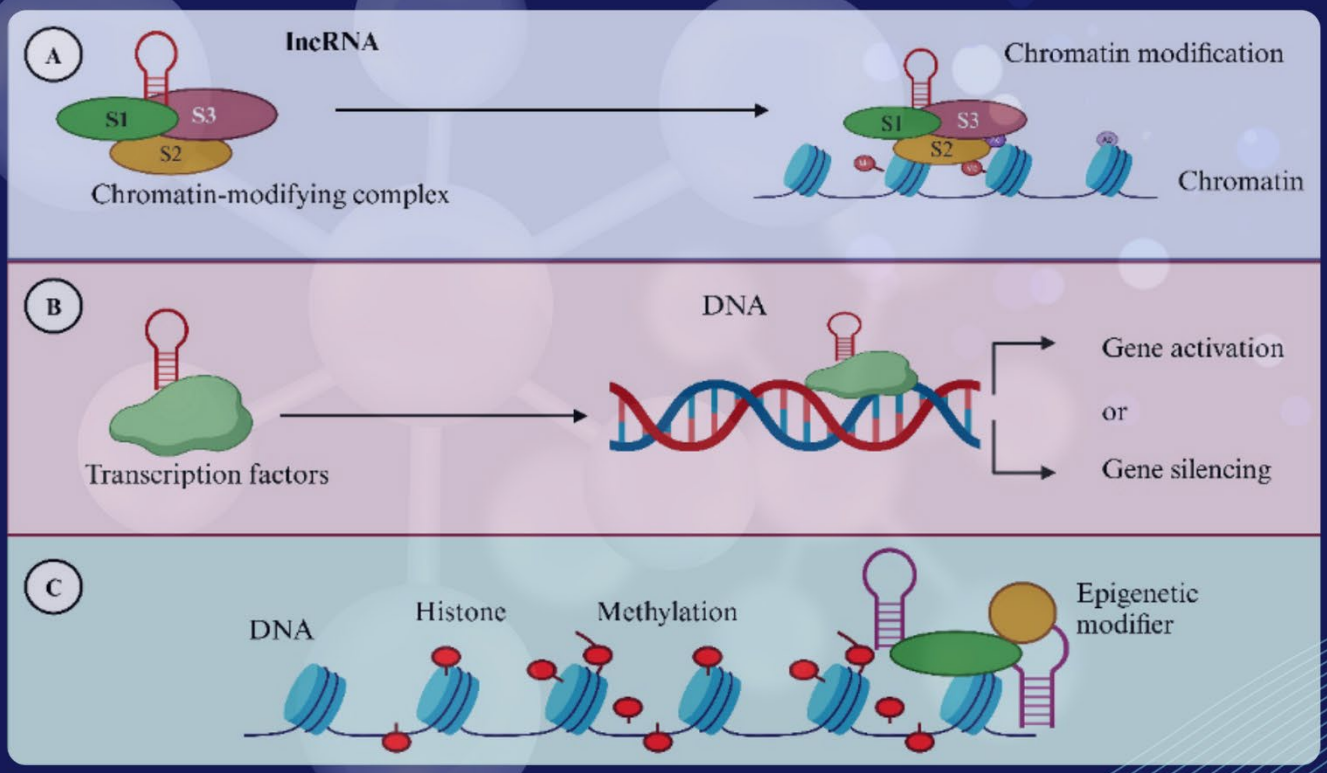

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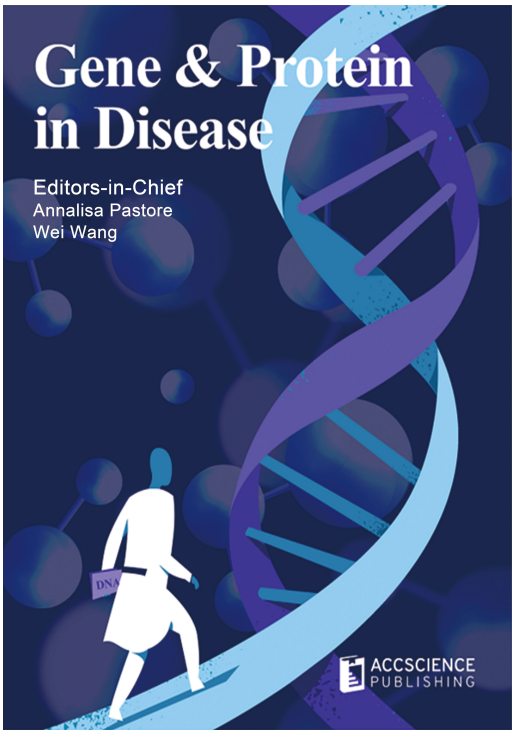

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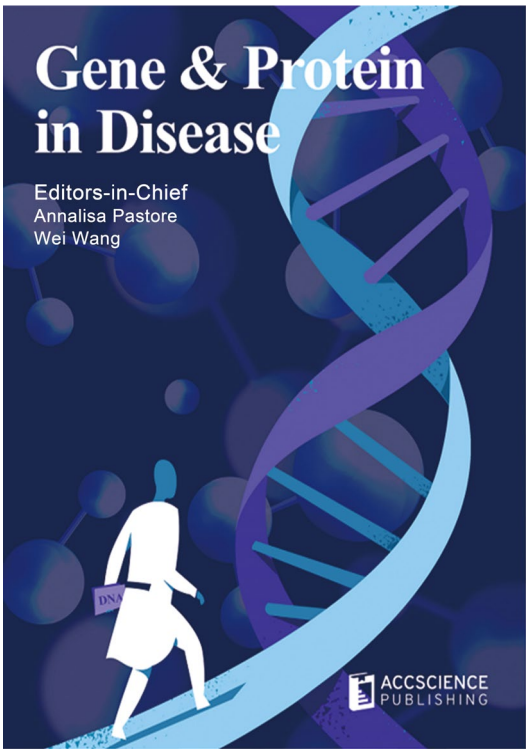

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A graphic illustration of double-stranded DNA

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REVIEW ARTICLE

Promoting stem cells activity using Chinese medicine herbs in treatment of neurological disorder: A review on novel therapeutic approaches

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Abstract

Stem cell therapy represents a burgeoning and swiftly advancing modality for the treatment of a diverse array of neurological disorders. However, despite continued clinical trials, the underlying mechanisms of action often remain elusive. Traditional Chinese medicine (TCM), with its holistic approach, provides a valuable resource for the identification and evaluation of potential neuroprotective agents. Research has shown that TCM, including herbs, herbal extracts, and specific Chinese herbal constituents, can modulate the proliferation and differentiation of neural stem cells (NSCs) to some extent. This review examines the potential of TCM as a treatment for neurodegenerative diseases. Given the limitations of current therapies due to a lack of understanding of disease pathogenesis, a holistic approach to TCM offers a promising alternative. This paper also summarizes the role of stem cells in the management of neurological disorders and evaluates prior studies concerning stem cell transplantation. In addition, it explores the capacity of TCM to influence the proliferation and differentiation of NSCs. The ultimate aim of this review is to enhance our understanding of how TCM can be utilized to influence stem cell behavior and potentially treat neurodegenerative diseases.

Keywords: Stem cell; Traditional Chinese medicine; Neurodegenerative diseases; Neuroprotective agents

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1. Introduction

Therapeutic strategies aimed at addressing neurological disorders that impact the central nervous system (CNS) are frequently constrained by our insufficient comprehension of pathophysiology. While certain CNS disorders have a genetic basis or are associated with specific proteins, a significant number entail intricate interactions among diverse cellular types and metabolites within the cerebral microenvironment.[1,2] CNS pathologies and

traumas manifest across diverse cell types, encompassing embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs). Experimental investigations conducted *in vitro*, *in vivo*, and through clinical trials are undertaken to elucidate the therapeutic efficacy for a range of neurological afflictions.[3,4]

The research and assessment of stem cells alongside various delivery methodologies are currently underway to evaluate their potential application in clinical settings pertaining to amyotrophic lateral sclerosis (ALS) and additional neurodegenerative disorders, including Alzheimer's disease (AD), Huntington's disease, Parkinson's disease (PD), and cerebrovascular accidents.[5,6] Previous investigations have demonstrated that the introduction of stem cells into animal models afflicted by neurodegenerative disorders has the potential to enhance the morphology of existing neurons. This process promotes the substitution of novel cells, which subsequently release trophic factors and modulate inflammatory responses.[7,8] Two distinct categories of stem cell-based therapeutic interventions have been delineated to date: the first category encompasses the stimulation or enhancement of endogenous neural progenitor cells to facilitate an augmented secretion of trophic factors and growth molecules conducive to tissue regeneration, while the second category is centered on the transplantation of exogenous stem cells.[9,10] Since many medications are not very effective and may induce side effects, the development of new drugs based on phytochemicals is gaining attention.[11]

Traditional Chinese medicine (TCM), a major alternative medicine, is widely practiced in Chinese communities and accepted in Western medicine. While the exact origins of TCM are unknown, archeological discoveries revealed that acupuncture needles and a few herbs have been applied for approximately 4000 – 8000 years.[12] TCM's holistic approach to neurodegenerative diseases leverages the synergistic effects of multiple herbal components to target systemic organs. For instance, herbs such as curcumin and resveratrol are used to improve memory,[13] while quercetin is believed to enhance cognitive function.[14] Research has demonstrated that monomers, botanical extracts, or combinations of Chinese herbal monomers, as well as instruments utilized in traditional Chinese medicine, possess the capability to modulate stem cell proliferation and differentiation to a certain degree.[15,16]

The primary aim of this scholarly article is to enhance the body of research pertaining to the application of TCM in the modulation of stem cell proliferation and differentiation within the context of neurodegenerative disorders. In pursuit of this objective, the article elucidates the implications of TCM on stem cell behavior.

2. Application of stem cell in treatment of neurologic disorders: advantages and disadvantages

Stem cell-based therapy, a key aspect of regenerative medicine, has garnered significant attention due to its potential to provide new treatment options for patients with previously untreatable diseases. As a result, thousands of clinical trials have been initiated, addressing a wide range of medical issues, including musculoskeletal and neurological disorders, immune diseases, blood disorders, and degenerative conditions.[17] However, some trials have not demonstrated clinical benefits, likely due to inherent limitations of stem cell therapy. These limitations include infusion toxicity, immunogenicity, tumorigenic potentials, and ethical concerns.[18]

Stem cells are unspecialized cells present during the embryonic, fetal, and adult phases of life, which develop into specialized cells that make tissues and organs. In post-natal and adult stages, tissue-specific stem cells reside within differentiated organs and play a key role in repairing injuries. The key characteristics of stem cells are potency, which allows them to differentiate into multiple cell types. These traits can vary among different stem cells. For instance, ESCs derived from the blastocyst stage exhibit greater self-renewal and potency, while adult tissue stem cells have restricted self-renewal capability and can only differentiate into specific tissue cells.[19]

Among the various types of stem cells, pluripotent stem cells (PSCs) are distinguished by their exceptional capacity to divide and differentiate into any cell type within an organism, including embryonic structures, but are not capable of forming extra-embryonic structures like the placenta; examples include ESCs derived from preimplantation embryos and iPSCs, which are generated from adult cells reprogrammed to an embryonic-like state. PSCs vary in their potency, ranging from fully pluripotent cells to those with reduced potency, such as multi-, oligo-, or uni-potent cells. iPSCs, created from somatic cells, exhibit similar function to PSCs and offer significant potential for regenerative medicine. Their activity is often assessed using the teratoma formation assay.[20,21]

Recent advancements in stem cell biology have enabled the differentiation of PSCs, such as ESCs and iPSCs, into neural progenitor or NSC-like cells for therapeutic purposes. NSCs, being the most logical choice for treating neurological disorders, are unfortunately located deep within the adult brain, making them inaccessible for harvesting and autologous therapeutic applications.[22] One significant advantage of PSCs is their ability to proliferate indefinitely, allowing for the production of billions of various

human cells for transplantation. However, this property is a double-edged sword, as uncontrolled proliferation post-transplantation can lead to tumor formation, with even a few residual PSCs potentially causing teratomas. Because PSCs have a high capacity for rapid growth and a risk of forming tumors, they are not used directly in treatments. Instead, they are differentiated into specific, fully developed cell types before being utilized.[23]

Numerous preclinical and clinical investigations have examined the safety, effectiveness, and practical application of diverse types of stem cells in the therapeutic management of neurological conditions, including AD,[24] PD,[25,26] multiple sclerosis (MS),[27] cerebrovascular accident,[28,29] Huntington's disease,[30] and spinal cord injury.[31,32] So far, only a few small-scale clinical trials have examined the safety and practicality of using stem cell therapies in patients diagnosed with AD. A Phase I trial utilizing MSCs derived from human umbilical cord blood indicated that repeated intravenous infusions were both safe and well-tolerated, with a noted stabilization of cognitive function among participants.[33] A separate Phase I clinical trial tested autologous adipose-derived mesenchymal stem cells. The trial showed that these cells are safe and may help reduce cognitive decline in people with mild to moderate AD.[34] The preliminary trials have limitations due to a small number of participants, insufficient control groups, and short follow-up periods. These factors make it challenging to draw definitive conclusions about the effectiveness of stem cell therapies for AD.[35]

Many clinical studies have evaluated the safety and effectiveness of stem cell therapies for people with PD.[26] Initial research using fetal ventral mesencephalic tissue grafts showed mixed results. Some patients experienced long-lasting improvements, while others developed movement disorders due to the grafts.[36,37] Recent studies using dopaminergic progenitors derived from human embryonic stem cells have shown promising results. A Phase 1/2 clinical trial verified that the transplanted cells are safe and effective, and some improvements in motor function were observed.[38] Separately, a Phase 1 clinical trial is currently in progress to assess the safety and efficacy of these cell-based treatments.[39] Furthermore, clinical investigation using autologous iPSCs to generate dopaminergic neurons is currently being planned.[40]

Many clinical studies have examined the safety and effectiveness of stem cell treatments for people with MS.[41,42] Autologous hematopoietic stem cell transplantation (aHSCT) is being investigated as a potential treatment for severe forms of MS, aiming to reset the immune system and slow the progression of disease. Compared to traditional disease-modifying therapies, aHSCT has shown significantly better results in preventing disease progression and improving neurological function. Long-term follow-up studies indicate that many patients remain free of disease activity for more than five years after undergoing this treatment.[43] Although the results are promising, the procedure carries significant risks and is intended for individuals with highly active disease.[44,45] Clinical trials that investigate the safety and feasibility of MSCs have also been conducted.[46] Administering autologous MSCs through an intravenous injection is generally well-tolerated and shows promise in lowering inflammation and enhancing neuroprotection.[47,48] However, larger randomized controlled trials are necessary to verify the long-term safety and effectiveness of MSC-based therapies in treating MS.

3. Herbal ingredients of Chinese medicine and their neurogenic effects

Having been utilized and practiced continuously for millennia, TCM offers a rich repository of herbal remedies known for their therapeutic benefits.[12] Among these, certain herbal ingredients have garnered attention for their neurogenic effects, which are crucial for promoting the growth, development, and regeneration of nervous tissue. Herbs such as *Ginkgo biloba*,[49] *Rhodiola*,[50] and ginsenoside Rg1[51] have been researched for their potential to promote neurogenesis, synaptogenesis, and overall brain plasticity. These properties make them promising candidates for the treatment and management of neurological disorders, including AD and other forms of cognitive decline.

3.1. Curcumin

Curcumin is a molecule derived from *Curcuma longa* as a polyphenolic ingredient.[52] Curcumin reduces the release of stimulated glutamate from synaptosomes in the rat prefrontal cortex by inhibiting presynaptic voltage-gated N-type CaV1.2 and CaV1.2 channels. This effect is comparable to that of the antidepressant fluoxetine, potentially by preventing glutamate release from nerve terminals.[53] J147, a derivative of curcumin, is a powerful drug candidate with neurogenic and neuroprotective properties. Originally developed to treat neurodegenerative diseases related to aging, this derivative affects multiple pathways involved in the development of diabetic neuropathy.[54] Curcumin effectively prevents oxidative stress, reduces mitochondrial dysfunction, and prevents nerve cell death caused by the loss of trophic support in cell culture models.[55,56] It reverses cognitive decline in a mouse model of AD and improves memory in both genetically modified AD mice and older wild-type mice. In addition, J147 helps reduce inflammation and slows down age-related metabolic decline in older mice, and exhibits neurogenic properties.[57,58]

3.2. Quercetin

Quercetin widely presents as a flavonoid in vegetables and fruits, and several studies have associated it with various neuroprotective processes, especially in connection with the role of microglial cells and astrocytes.[59,60] It has been shown that quercetin inhibits cell proliferation at high doses but promotes the formation of new neurons at lower doses.[61] Specifically, 25 μM of quercetin enhanced exit from cell-cycle and supported the survival and differentiation of adult hippocampal neural progenitor cells, both in lab settings and in living organisms.[62] It has also been demonstrated that quercetin effectively prevents the impairments caused by high glucose and methylglyoxal, as well as the neurogenic-mediated relaxations of mouse corpora cavernosa.[63]

3.3. Resveratrol

Resveratrol, found in plants like red grapes, is known for its ability to protect against neurodegenerative diseases.[64,65] It has been demonstrated to promote the growth of new neurons and the formation of new mitochondria by enhancing AMPK activity, without relying on SIRT activation.[66] In a C57B1/6 mouse model with streptozotocin-induced diabetes, resveratrol administration restored normal levels of neurogenesis and synaptic plasticity. This effect was mediated by SIRT1 and AMPK. Furthermore, a comprehensive gene expression analysis showed that resveratrol normalized the expression of genes associated with hippocampal neurogenesis and synaptic plasticity, such as *Hdac4*, *Hat1*, *Wnt7a*, and *ApoE*.[67] In addition, animals treated with resveratrol showed enhancements in neurogenesis and in the hippocampus affected by microvascular culture. In addition, resveratrol reduced the enlargement of astrocytes and the activation of microglia in this brain region.[68]

3.4. Ginsenoside

Ginsenosides enhance neurogenic vasodilation in the corpus cavernosum. The relaxation of the corpus cavernosum by ginsenosides is primarily mediated by nitric oxide (NO) and may partly explain the aphrodisiac effects of *Panax ginseng*.[69] Furthermore, ginsenoside Rb1, a major component of *Panax ginseng*, has been demonstrated to alleviate depressive-like behaviors in male mice subjected to chronic mild stress primarily by activating microglia through PPARγ and enhancing neurogenesis in the adult hippocampus.[70]

4. Combination of stem cell and herbal ingredients

In TCM, the primary active substances are small molecules, which can effectively target stem cells.[71] These molecules are known for their rapid, dose-dependent biological effects, as well as their accessibility and ease of use. TCM compounds can enhance stem cell functions, such as delaying aging or stimulating tissue regeneration. Small molecules derived from TCM have shown potential in improving stem cell renewal, differentiation, proliferation, and survival in regenerative medicine.[72,73] In the following sections, we describe some of the most commonly used Chinese herbal medicine and their effect on stem cells which are summarized in Table 1.

4.1. Curcumin

It has been shown that curcumin can protect stem cells from oxidative stress and improve stem cell proliferation and survival in a dose-dependent manner. For example, low concentrations of curcumin promote the proliferation of bone marrow-derived mesenchymal stem cells (BMSCs) and promoting osteogenic differentiation.[55,74,75] In addition, curcumin has also been shown to regulate inflammatory signaling pathways, lending credence to its potential in regenerative medicine. However, high concentrations may have toxic effects, underscoring the importance of appropriate dosing in regenerative medicine.[76,77] In addition to its effects on BMSCs, curcumin has been shown to promote the differentiation of NSCs into neurons, while reducing oxidative stress, which is a critical factor in neuroprotection.[71] These properties make curcumin a valuable compound in regenerative medicine, especially for neurodegenerative conditions where enhanced neurogenesis and neuroprotection are needed.

4.2. Quercetin

Quercetin has shown significant effects on stem cell biology, particularly in BMSCs. Studies indicate that quercetin promotes the differentiation and proliferation of BMSCs by activating the Wnt/β-catenin pathway and estrogen receptor-mediated mechanisms. In addition, the stimulative effects of quercetin on mineralization of the extracellular matrix and osteogenic markers expression such as BMP-2, Runx2, and osterix have been approved.[78] Given these effects, quercetin can be utilized as a particularly effective therapeutic approach to increase stem cell efficacy and proliferation for clinical applications.[79-81] Beyond its effects on BMSCs, quercetin enhances NSC survival and differentiation, particularly by reducing neuroinflammation.[82] This effect supports its potential use in regenerative therapies for brain injuries and neurodegenerative diseases, where neuroinflammation is a major concern.

4.3. Aucubin

Aucubin, an iridoid glycoside, influences NSCs by promoting neurite outgrowth and differentiation, which

Table 1. Traditional Chinese medicine compounds with neurogenic effects on stem cells

Herbal compound	Study focus	Key findings	References
Curcumin	Neuroprotection and neurogenesis	• Improves differentiation of neural stem cells into neurons • Reduces oxidative stress	71
Quercetin	Anti-inflammatory and neuroprotective effects	• Improves survival and differentiation of neural stem cells • Ameliorates neuroinflammation	82
Aucubin	Neuroprotection	• Increases proliferation and differentiation of neural stem cells • Enhances protection against neurotoxicity	83, 84
Baicalin	Neurogenesis and neuroprotection	• Increases proliferation and differentiation of neural stem cells • Reduces apoptosis	80, 88
Ginkgolide B	Neuroprotection	• Promotes survival and differentiation of neural stem cells • Relieves oxidative stress and inflammation	90, 91
Ginsenoside Rg1	Neurogenesis and neuroprotection	• Increases proliferation and differentiation of neural stem cells • Enhances neurogenesis	92, 94
Resveratrol	Neuroprotection and anti-aging	• Enhances survival and differentiation of neural stem cells • Ameliorates oxidative stress and inflammation	97
Tanshinone IIa	Neuroprotection and anti-inflammation	• Increases proliferation and differentiation of neural stem cells • Reduces neuroinflammation	100
Astragaloside IV	Neuroprotection and neurogenesis	• Increases proliferation and differentiation of neural stem cells • Decreases oxidative stress	104
Astraisoflavan	Neuroprotection	• Enhances survival and differentiation of neural stem cells • Minimizes neurotoxicity	73

may be beneficial for neuroregeneration. The previous studies approved that aucubin has osteogenic effects and promotes hippocampal NSCs differentiation into neurons and their proliferation by increasing the expression of neuronal markers such as NeuN and MAP2. The beneficial effects of aucubin have largely been linked to its ability in reducing oxidative stress and inflammation resulting in a conducive microenvironment for neuronal regeneration. Therefore, this herbal compound can attract the attention of scientists as a novel stem cell-based therapeutic strategy.[83,84]

4.4. Baicalin

Baicalin is a bioactive flavonoid isolated from *Scutellaria baicalensis*, which can significantly influence activity of stem cells.[85,86] Its effects on stem cell differentiation have been widely studied, particularly in dental pulp stem cells and neural stem/progenitor cells.[80,87] This inhibition results in an increased expression of osteogenic markers and enhanced mineralization, which is vital for bone and tissue regeneration. In addition, a study has demonstrated that baicalin promotes the neuronal differentiation of neural stem/progenitor cells, stimulating neurogenesis and improving cognitive function following ischemic injury.[88] These findings highlight the potential of baicalin in regenerative medicine, particularly for the therapeutic repair and regeneration of dental and neurological tissues.[80,88]

4.5. Ginkgolide B

Ginkgolide B, a key active compound in *Ginkgo biloba* extract (GBE), has been shown to influence the differentiation of various stem cell types. In NSCs, Ginkgolide B promotes cell cycle exit and differentiation into neurons through the activation of the Wnt/β-catenin signaling pathway.[89] Moreover, Ginkgolide B has been found to support *in vitro* differentiation of NSCs into dopaminergic neurons. However, Ginkgolide B also exerts cytotoxic effects on blastocyst-stage embryos in a dose-dependent manner, inducing apoptosis during early embryonic development. Despite these findings, the precise mechanisms and therapeutic potential of Ginkgolide B in stem cell differentiation require further investigations to better understand its benefits in stem cell biology.[90,91]

4.6. Ginsenoside Rg1

Ginsenoside Rg1, a bioactive compound derived from ginseng, has been shown to promote the differentiation and proliferation of stem cells from various origins.[92,93] Several studies have demonstrated that ginsenoside Rg1 enhances the osteogenic and chondrogenic differentiation of bone marrow-derived mesenchymal stem cells by activating the Wnt/β-catenin signaling pathway, which involves the inhibition of glycogen synthase kinase 3 beta (GSK-3β) phosphorylation. In addition, ginsenoside Rg1 has protective effects against stem cell senescence.[93,94] While ginsenoside Rg1 is known to promote osteogenic

differentiation in BMSCs, it also enhances neurogenesis and differentiation in NSCs, providing neuroprotection and promoting neuronal survival.[93,94] These combined effects underline ginsenoside Rg1's potential as a therapeutic agent across multiple stem cell types in regenerative medicine.

4.7. Resveratrol

Recent studies have shown that resveratrol promotes osteogenic differentiation of BMSCs by upregulating alkaline phosphatase activity[95] and enhancing mineralization, alongside the promotion of mitochondrial biogenesis and a shift in energy metabolism toward differentiation.[96] Alongside promoting osteogenic differentiation in BMSCs, resveratrol supports NSC survival and differentiation by reducing oxidative stress and inflammation, making it particularly relevant in neuroprotection and anti-aging therapies.[97] Its ability to modulate the SIRT1-ERK pathway also offers protective effects against reactive oxygen species, which is crucial in both stem cell and neural cell longevity.[97] As a result, resveratrol holds promise as a therapeutic agent in various regenerative medicine.

4.8. Tanshinone IIA

Tanshinone IIA is a bioactive component isolated from *Salvia miltiorrhiza* that has been found to considerably support differentiation and proliferation of stem cells, primarily human BMSCs.[98] Recent studies have indicated that Tanshinone IIA enhances BMSC expansion by upregulating fibroblast growth factor 2 and activating the PI3K/AKT signaling pathway, which is crucial for cell cycle progression and survival.[99] In addition to its beneficial effects on BMSC proliferation, Tanshinone IIA also promotes neuroprotection by supporting the proliferation and differentiation of NSCs, reducing neuroinflammation, and aiding in neuronal recovery after ischemic injuries.[100] This dual role makes Tanshinone IIA valuable for applications in both bone and nervous system repair.

4.9. Astragaloside IV

Astragaloside IV, a bioactive compound extracted from *Astragalus membranaceus*, has shown significant effects on stem cell functionality and differentiation. Recent studies indicate that Astragaloside IV can enhance the proliferation and survival of BMSCs under challenging conditions such as iron overload and oxidative stress.[101,102] Astragaloside IV has also shown a capacity to induce angiogenesis from adipose-derived mesenchymal stem cells through activating the FAK phosphorylation signaling pathway, with a targeted effect on ischemic diseases.[103] While Astragaloside IV supports BMSC survival and angiogenesis, it also enhances NSC proliferation and differentiation, reducing oxidative stress and promoting neural regeneration.[104] Its multiple functions across different stem cell types enhance its therapeutic potential for a range of regenerative applications. Furthermore, in another experimental study, Astragaloside IV reduces the breast cancer stem cell, increasing sensitivity to paclitaxel as a chemotherapy agent.[101] In addition, the cardioprotective effects of Astragaloside IV exerted through the induction of BMSC-derived exosomes have been proven in mouse models of acute myocardial infarction.[105] Overall, Astragaloside IV is regarded as an agent with multiple effects on stem cell biology, possessing potential applications for regenerative medicine and cancer interventions.[105]

4.10. Astraisoflavan

Astraisoflavan is a biologically active compound derived from *Astragalus membranaceus* that has demonstrated various effects in stem cell biology, especially NSCs.[106] Recent studies provided evidence that astraisoflavan increases NSC proliferation and dopamine neuron differentiation, which is indispensable for neurodegenerative diseases like PD.[107] These effects appear to involve the upregulation of several important transcription factors, such as pituitary homeobox (Ptx3), sonic hedgehog (Shh), and orphan nuclear hormone 1 (Nurr1), which are important for dopamine neuron development. In addition, astraisoflavan increases the expression of prominent markers of dopaminergic neurons, including the dopamine transporter (DAT) and tyrosine hydroxylase (TH).[106] However, more studies need to be conducted to determine the underlying various molecular mechanisms of astraisoflavan on the stem cells, and its possible uses as a novel therapeutic approach against neurodegenerative disease.

In summary, BMSCs and NSCs both play essential roles in tissue regeneration due to their differentiation and self-renewal capabilities. While NSCs directly regenerate nerve tissue by forming neurons and glial cells, BMSCs provide a supportive environment by secreting factors that enhance neurogenesis and neuroprotection. This complementary relationship supports their combined use in regenerative therapies for nervous system repair.

5. Conclusion

The combination of stem cell therapy and TCM herbal ingredients offers a promising approach for treating neurological disorders. Stem cells provide the potential to replace damaged neural cells and restore lost functions, while bioactive compounds from TCM – such as curcumin, resveratrol, and ginsenosides – can modulate stem cell behavior to promote neurogenesis, enhance neuroprotection, and create a supportive regenerative environment. This synergy may improve treatment

efficiency, minimize side effects, and pave the way for safer and more effective therapies. However, challenges remain, including potential immunogenicity, tumorigenic risks, and dosage sensitivity for both stem cells and TCM ingredients. To harness these benefits and translate them into clinical practice, further research should focus on refining combined therapy protocols, optimizing dosage and delivery methods, and conducting large-scale trials with rigorous safety and efficacy assessments. Future studies should also establish clear patient selection criteria and evaluate long-term outcomes to ensure clinical applicability and improved patient care in the field of neuroregenerative medicine.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Current insights and advances in long noncoding RNA dysregulation in diabetes and its complications

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Abstract

Long noncoding RNAs (lncRNAs) are pivotal regulators in the pathophysiology of diabetes mellitus and its complications, including diabetic retinopathy, nephropathy, and cardiomyopathy. They influence essential cellular processes such as angiogenesis, vascular smooth muscle cell behavior, inflammation, extracellular matrix remodeling, and apoptosis. In cardiomyopathy, lncRNA H19 promotes cardiomyocyte survival by regulating autophagy, highlighting its role in cardiovascular outcomes. In addition, lncRNAs hold promise as noninvasive biomarkers, detectable in extracellular fluids such as serum and urine, providing novel diagnostic tools. For example, elevated levels of HOX transcript antisense RNA and promoter of CDKN1A antisense DNA damage-activated RNA correlate with hyperglycemia and its complications, whereas maternally expressed gene 3 and metastasis-associated lung adenocarcinoma transcript 1 are linked to insulin resistance. However, the full scope of lncRNA mechanisms and therapeutic potential remains unclear. Recent research emphasizes the importance of studying the molecular pathways influenced by lncRNAs, such as the Janus kinase/signal transducer and activator of transcription and p38 mitogen-activated protein kinase pathways, which regulate inflammation and insulin signaling. Preclinical studies show promising outcomes for lncRNA-targeted therapies; however, challenges remain in precise detection and delivery. Addressing these gaps through advanced RNA sequencing and targeted therapies is crucial for developing lncRNA-based diagnostics and treatments for diabetes complications. This review explores lncRNAs as biomarkers and therapeutic targets, focusing on their regulatory mechanisms in diabetic complications such as retinopathy, nephropathy, and cardiomyopathy.

Keywords: Diabetes mellitus; Long noncoding RNA; Biomarkers; Transcriptional control; Insulin resistance; Vascular problems

1. Introduction

Noncoding RNAs (ncRNAs) exceeding 500 nucleotides, known as long ncRNAs (lncRNAs), represent a distinct type of RNA transcript, transcribed from DNA but not translated into proteins. Numerous recent studies have confirmed that lncRNAs play crucial regulatory roles in nearly all biochemical processes and pathways.[1] ncRNAs are classified by length into three main categories: small RNAs, which are fewer than 50 nucleotides long; RNA polymerase III transcripts, ranging from approximately 50 to 500 nucleotides; and lncRNAs, which are typically over 200 nucleotides. LncRNAs are further categorized by their origin and characteristics. They may be intergenic, antisense, intronic, or derived from pseudogenes. Some lncRNAs resemble messenger RNAs (mRNAs), being spliced and polyadenylated, whereas others lack polyadenylation or a 7-methylguanosine cap. In addition, circular RNAs formed by backsplicing and trans-acting regulatory RNAs are classified as lncRNAs.[2] In transcription, posttranscriptional regulation, and epigenetics, lncRNAs influence gene expression. Current research shows that neurological, endocrine, and metabolic disorders are closely linked with lncRNAs.[3] Although mRNAs are expressed at higher levels than most lncRNAs, many lncRNAs play key roles in regulating cellular homeostasis and gene expression.[4] Due to their abnormal activities in controlling certain biological processes and their dysregulated expression in various diseases, lncRNAs have drawn significant attention. Studies indicate substantial variation in the number of noncoding genes between species, suggesting that this variation is related to organismal complexity.[4,5] Understanding how lncRNAs regulate transcriptional or posttranscriptional processes, as shown in Figure 1, enhances our knowledge of disease.

Diabetes mellitus (DM) is the most common metabolic condition, categorized by a lack of insulin release, impaired insulin action, or both.[6] The global prevalence of DM presents a significant public health challenge due to the substantial financial burden it places on both patients and society. A thorough understanding of DM pathophysiology is crucial for developing more effective preventive and therapeutic strategies. Routine evaluation, early diagnosis, and efficient management of chronic complications are vital for reducing the morbidity and mortality associated with DM.[7,8] Advances in modern technologies have identified numerous lncRNAs as novel regulators in the pathophysiology of DM, potentially offering new approaches for treatment, early diagnosis, and prevention. Clinical phenotypes have revealed several correlations between altered gene expression and lncRNA physiology. LncRNAs play key roles in regulating insulin production, β-cell death, glucose metabolism, and insulin resistance.[9-11] Consequently, various studies have reported changes in the expression of lncRNAs related to type 1 DM (T1DM) and type 2 DM (T2DM) in murine models.[11] In this context, lncRNAs may serve as valuable biomarkers for the early detection of, and susceptibility to, T1DM or T2DM.[11,12] For example, Carter *et al*. (2015) found that growth-arrest-specific transcript 5 (*GAS5*) may be a predictive biomarker for T2DM, as this lncRNA was reduced in the serum of DM patients within a cohort of USA military veterans.[13] Individuals with reduced *GAS5* expression were nearly 12 times more likely to have T2DM.[14]

Current research lacks comprehensive insights into how specific lncRNAs contribute directly to diabetes-associated complications. Our review bridges this gap by identifying various lncRNAs that regulate angiogenesis and vascular cell stability, offering new directions for investigating their roles in diabetes-induced complications. Furthermore, validating specific lncRNAs as reliable biomarkers for DM and its complications is crucial, highlighting their potential as noninvasive diagnostic tools and therapeutic targets. This gap also underscores the need for improved delivery methods and robust clinical trials to address challenges in translating these findings into effective clinical applications. Here, we examine the biological processes involving lncRNAs in DM, their significant roles in DM, and summarize the history and general functions of lncRNAs. In addition, we explore how lncRNAs can function as biomarkers for early diagnosis, prevention, and treatment of DM.

2. LncRNAs in pancreatic β-cells

Pancreatic β-cells primarily produce and secrete insulin, a hormone that promotes glucose uptake by cells to regulate blood glucose levels. Exonic readings from proinsulin mRNA in pancreatic islets were observed 20% of the time, whereas this percentage rose to 45% in β-cells, according to a transcriptome study.[15] In recent years, islet lncRNA*s* have emerged as key regulators of insulin synthesis and release, although it has long been known that several islet-enriched transcription factors tightly control insulin gene expression. The imprinted noncoding gene known as the maternally expressed gene (*Meg3*) is located on mouse chromosome 12, with its human homolog on chromosome 14. Although *Meg3* shows higher expression in normal tissues, it is downregulated in many human cancers or tumor-derived cell lines.[14] *Meg3* expression within the islet of Langerhans is 20 times higher in human β-cells than in α-cells, which produce glucagon, suggesting a specific role in β-cells. Glucose regulates *Meg3* expression dynamically, and blocking it with small interfering RNAs (siRNAs) in murine insulinoma cell line 6 (MIN6) mouse β-cells leads

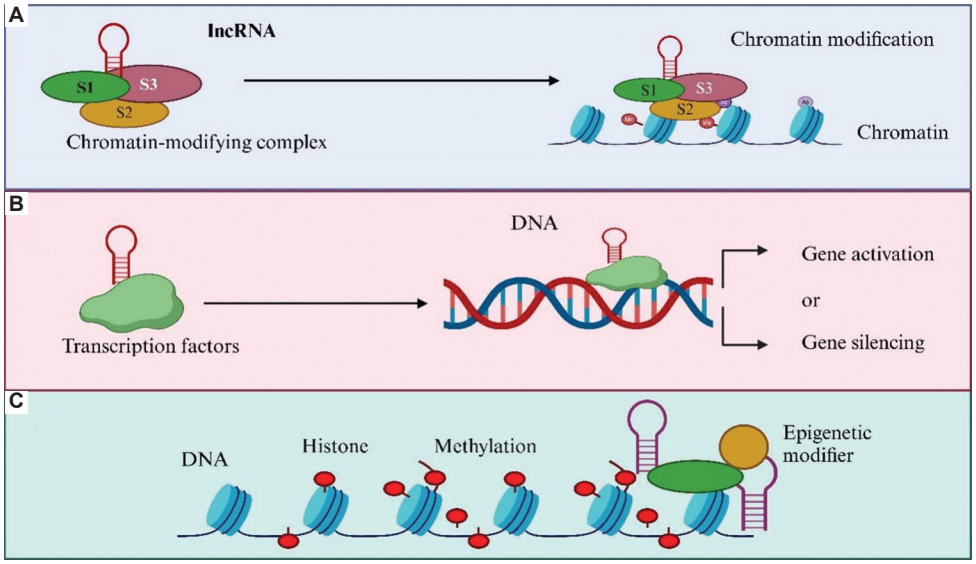

Figure 1. Regulatory roles of long noncoding RNAs (lncRNAs) in gene expression. (A) LncRNAs regulate gene expression by interacting with chromatin-modifying complexes, influencing chromatin structure and transcriptional activity. (B) They bind to DNA, histones, and transcription factors, modulating chromatin accessibility to activate or silence genes. As enhancers, lncRNAs play a key role in regulating genes and determining cellular identity by modulating enhancer activity, affecting target gene expression, and driving cell differentiation and development. (C) LncRNAs contribute to epigenetic modifications, such as DNA methylation and histone changes, shaping gene expression patterns and cellular traits. They also facilitate biomolecular condensate formation through phase separation, organizing gene expression spatially during development. Moreover, lncRNAs regulate protein translation, localization, and stability, influencing cellular processes, such as metabolism and signaling.

to increased β-cell death and reduced insulin production and secretion. Moreover, Balb/c female mice treated with intravenous *Meg3*-targeting siRNAs expressed and secreted less insulin. Compared to control animals, these mice's islets exhibited lower mRNA levels of Ins2, pancreatic and duodenal homeobox 1 (PDX1), and musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*). *Meg3*'s nuclear presence in pancreatic β-cells suggests a role in transcriptional regulation, though the precise mechanisms by which it controls insulin synthesis remain unknown. *Meg3* has been shown to regulate gene expression in various tissues by recruiting the histone methyltransferase polycomb repressive complex 2 (PRC2).[14]

The islets of Langerhans contain highly specialized endocrine cells primarily responsible for storing and releasing insulin in response to glucose levels. The pathophysiology of both T1DM and T2DM is affected by alterations in cellular identity or function. Recent studies indicate that *lncRNAs* regulate various biological functions in islets, including cell differentiation, proliferation, insulin production, and secretion. Morán *et al.* (2012) mapped the transcriptome and identified 1128 genes for human pancreatic islets, with islet-specific lncRNAs representing 55% of long intergenic ncRNAs (lincRNAs) and 40% of antisense lncRNAs.[15] Ku *et al.* (2012) used RNA sequencing to identify over 1000 lncRNAs or lincRNAs in mouse islets, most of which affect only certain cells. Notably, lincRNA-XLOC 019089 was exclusively found in a specific tissue and was antisense to the *Pdx1* gene in the pancreas and duodenum of mice.[16] Recently, deep RNA sequencing of isolated human cells from 11 healthy cadaveric islets identified 132 overexpressed lincRNAs compared to whole islets. In addition, 148 lncRNAs were overexpressed in specific cell types compared to others.[17] Many β-cell lncRNAs exhibit restricted distribution, suggesting that they perform highly cell-specific functions, including regulating the epigenetic environment and gene expression patterns that define cell types. Interestingly, islet lncRNAs are often located near islet-enriched or specialized genes involved in transcription, development, and cellular function.[15,16]

Researchers have demonstrated, using the quantitative polymerase chain reaction (qPCR) technique, that lncRNAs are activated at various stages of cell maturation.[15] Of the 13 cell-specific lncRNAs, all but one were inactive or minimally expressed in human embryonic pancreatic precursors before becoming functional islets in adults. In a model of differentiation using human embryonic stem cells, comparable results were observed: 12 lncRNAs were identified, with half either silent or expressed at very low levels during *in vitro* differentiation. However, these lncRNAs were significantly activated during *in vivo* maturation.[18] This indicates that islet lncRNAs play a crucial role in the differentiation and maturation of

β-cells and suggests that abnormal expression of β-cell-specific lncRNAs contributes to diabetes pathogenesis. Furthermore, HI-LNC45 expression was remarkably reduced, whereas KCNQ1 overlapping transcript-1 concentrations were significantly elevated in the islets of T2DM patients.[15] Further analysis of 55 T2DM susceptibility loci revealed that nine contained islet lncRNAs within 150 kb of the lead single nucleotide polymorphism, and six of these loci were directly linked to cellular dysfunction. Notably, islet lncRNAHI-LNC25 was found to potentially regulate GLIS family zinc finger protein 3 (GLIS3), closely associated with T2DM incidence.[15,19-21] These findings highlight islet lncRNAs as potential biomarkers for diabetes. Their proximity to islet-specific open chromatin regulatory clusters (COREs) implies possible coregulatory mechanisms with nearby protein-coding genes.[15] For example, Fadista *et al.* (2014) demonstrated that paired box 6 (PAX6), synaptotagmin 11 (SYT11), and the MAP kinase-associated death domain control proinsulin production, insulin exocytosis, and the formation of pancreatic islets *in vitro*. These genes were substantially co-expressed with the islet lncRNA LOC283177.[22] In light of these findings, further in-depth research on the functional role of lncRNAs in regulating diabetes diagnosis and pathogenesis is essential.

3. LncRNAs in the regulation of insulin secretion and sensitivity

Pancreatic β-cells respond to circulating nutrients, enabling the body to release insulin as needed. Diabetes primarily results from either absolute or relative insulin insufficiency. Consequently, modern diabetes treatments often focus on enhancing insulin sensitivity or secretion. Various lncRNAs play a role in regulating insulin secretion and sensitivity. For instance, steroid receptor RNA activator (*SRA*) has been found to enhance insulin sensitivity and promote glucose uptake by adipocytes in response to insulin stimulation.[23] The capacity of SRA to control the expression of multiple components affecting insulin sensitivity appears to play a key role in the underlying mechanisms. For instance, suppression of tumorigenicity 2 in adipocytes overexpressing SRA-like receptors led to a reduction in the expression of negative regulators of insulin sensitivity. Contrary to negative regulators, such as SH3 domain-containing 1, suppressors of cytokine signaling -1 and 3 promote the expression of positive regulators.[23] Researchers have also shown that SRA enhances insulin function, particularly glucose uptake, through the Akt and forkhead box O1 (FOXO1) signaling pathways. Knockdown of SRA using a lentiviral system resulted in the inhibition of insulin-stimulated glucose uptake and insulin-stimulated FOXO1 and Akt phosphorylation.[24]

Yin *et al.* (2015) found that downregulating the lncRNA taurine-upregulated gene (*TUG1)* increased cell death and reduced insulin secretion from islets both *in vitro* and *in vivo*.[25] In Min-6 cells, knocking down *TUG1* in mice led to decreased mRNA levels of transcription factors essential for insulin production and secretion, including glucose transporter 2 (GLUT2), (*MafA*), neurogenic differentiation 1 (NeuroD1), Pdx1, and NeuroD2.[25] During pregnancy, *Meg3* was identified as a novel lncRNA involved in insulin production and secretion, and its knockdown decreased insulin levels by suppressing key transcription factors such as Pdx1 and MafA.[25] Many lncRNAs likely target similar pathways to regulate insulin secretion.[26] In summary, the data suggest that lncRNAs have therapeutic potential in enhancing insulin sensitivity and secretion.

Hepatic insulin resistance is a hallmark of T2DM, as the liver plays a critical role in glucose and lipid metabolism. Insulin-stimulated Akt activation primarily inhibits glucose synthesis while promoting glycogen synthesis in hepatocytes.[27] Several ncRNAs, especially miRNAs, have been found to influence hepatic insulin signaling and contribute to the development of diabetes.

4. Role of lncRNAs in DM complications

Many diabetic complications are linked to microvascular and macrovascular dysfunctions, contributing to conditions such as retinopathy, nephropathy, and cardiovascular and peripheral vascular diseases.[28] Several of these complications have been associated with specific genes (Table 1). These vascular problems often feature impaired angiogenesis.[29,30] Various lncRNAs associated with diabetes have been shown to regulate angiogenesis individually, though only a limited number are directly connected to diabetic vascular complications. *In vivo* vascular development was inhibited by pharmacological and siRNA-induced silencing of metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*).[31] In addition, lncRNAs enriched in vascular cells, smooth muscle, and endothelial cells were initially identified in vascular smooth muscle cells (VSMCs) of coronary arteries.[32] Further research found that lncRNAs help maintain the stability of the contractile phenotype of VSMCs and inhibit VSMC migration.[32] Because VSMC development in response to hyperglycemia is a hallmark of diabetic cardiovascular complications,[33] researchers identified that lincRNA-p21 suppresses macrophage and VSMC growth while inducing cell death through p53 signaling.[34] This suggests that lincRNA-p21 contributes to diabetic vascular complications or related inflammation. Beyond MALAT1, which regulates inflammatory cytokine production and stimulates SAA3 in endothelial cells,[35] no additional experimental data on lncRNAs in diabetic vascular

Table 1. Summary of known interactions between specific lncRNAs and their target genes in diabetes

lncRNA	Target gene	Experimental model	Functions	Clinical relevance	References
HI-LNC25	*GLIS3*	Mice	HI-LNC25 promotes the expression of GLIS3 messenger RNA (mRNA), which contains type 1 and type 2 diabetes risk factors.	Clinically relevant for β-cell programming and diabetes pathophysiology for T2DM	15
lncLST	*ApoC2*	Mice	ncLSTR regulates the FXR/apoC2/PLP passage through TDP-43/Cyp8b1 to maintain systemic lipid homeostasis.	LncLSTR is a potential therapeutic target for hyperlipidemia and related complications	36
MALAT1	*CPNL1*	Rat	MALAT1 is potential MALAT1 is a potential biomarker for diabetes because it is markedly upregulated fibers and conducting cell membranes of diabetes-suffering patients, aqueous humor samples, and a hyperglycemic RF/6A cell line.	Increased TGF-β expression and its role in extracellular matrix production are implicated in the progression of diabetic nephropathy	37,38
PVT1	*FN1, COL4A1, TGFB1* and *PAI-1*	Human coronary artery smooth muscle cells	PVT1 has been linked to ESRD in T1DM and also in T2DM, most likely causing the kidney to accumulate an extracellular matrix.	SENCR is implicated in vascular smooth muscle cell phenotype regulation, with potential relevance to vascular diseases by influencing cell migration and stability	32
Lnc13	*STAT1 mRNA*	Human pancreatic islets	In an allele-specific manner, to sustain-cell inflammation, Lnc13-PCBP2 interactivity controls STAT1 mRNA secureness.	Lnc13 contributes to the pathogenesis of type 1 diabetes by increasing inflammation in pancreatic β-cells, linked to disease-associated SNPs	39

Abbreviations: ApoC2: Apolipoprotein C2; COL4A1: Collagen type IV alpha 1; CPNL1: Carboxypeptidase N, polypeptide 1; Cyp8b1: Cytochrome P450 8B1; ESRD: End-stage renal disease; FN1: Fibronectin 1; FXR: Farnesoid X receptor; GLIS3: GLIS family zinc finger 3; HI-LNC25: Hypoxia-inducible long noncoding RNA 25;lncLST: Long noncoding RNA LST; lncRNA: Long noncoding RNA; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; PAI-1: Plasminogen activator inhibitor-1; PLP: Phospholipid transfer protein; PVT1: Plasmacytoma variant translocation 1; RF/6A: Rat fetal retinal cell line; SENCR: Smooth muscle-enriched long noncoding RNA; STAT1: Signal transducer and activator of transcription 1; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; TDP-43: TAR DNA-binding protein 43; TGF-β: Transforming growth factor beta; TGFB1: Transforming growth factor beta 1.

complications have been reported. Nonetheless, these findings offer promising directions for future research.

4.1. LncRNAs and diabetic nephropathy (DN)

DN is a prevalent and severe complication of diabetes, significantly contributing to the global burden of chronic kidney disease.[40] It accounts for roughly 40% of end-stage renal disease cases.[41] Early indicators of DN include the buildup of extracellular matrix (ECM) proteins, such as collagen and fibronectin, glomerular mesangial cell (MC) expansion, hypertrophy, and podocyte effacement.[42,43] Clinically, albuminuria serves as a key biomarker for diagnosing and staging DN.[44,45] Alongside albuminuria, excessive ECM accumulation and thickening of the glomerular basement membrane, primarily in MCs, are distinct pathological features of DN (Figure 2). Genetic predispositions play a crucial role in DN susceptibility.[46,47] Recent studies also emphasize the role of epigenetic mechanisms – such as miRNAs, lncRNAs (Table 2), DNA methylation, and histone modifications – in DN pathogenesis.[48] Multiple molecular pathways, including inflammation, oxidative stress, the hexosamine biosynthetic pathway, and the polyol pathway, drive DN progression.[49] These factors, combined with ECM remodeling, contribute to declining renal function, eventually leading to kidney failure.

4.2. LncRNAs and diabetic retinopathy (DR)

DR is a chronic microvascular complication of DM that affects the retina due to prolonged hyperglycemia. Several lncRNAs are involved in DR pathogenesis. One notable lncRNA is the retina ncRNA 2 gene, also known as myocardial infarction-associated transcript (MIAT) in mice and gomafu in humans. MIAT is located on chromosome 22q12.1, a region linked to increased myocardial infarction risk.[56,57] Initially, MIAT was identified for regulating retinal cell proliferation in mammals.[58] In DR, MIAT is upregulated, contributing to retinal vascular dysfunction and worsening microvascular complications.

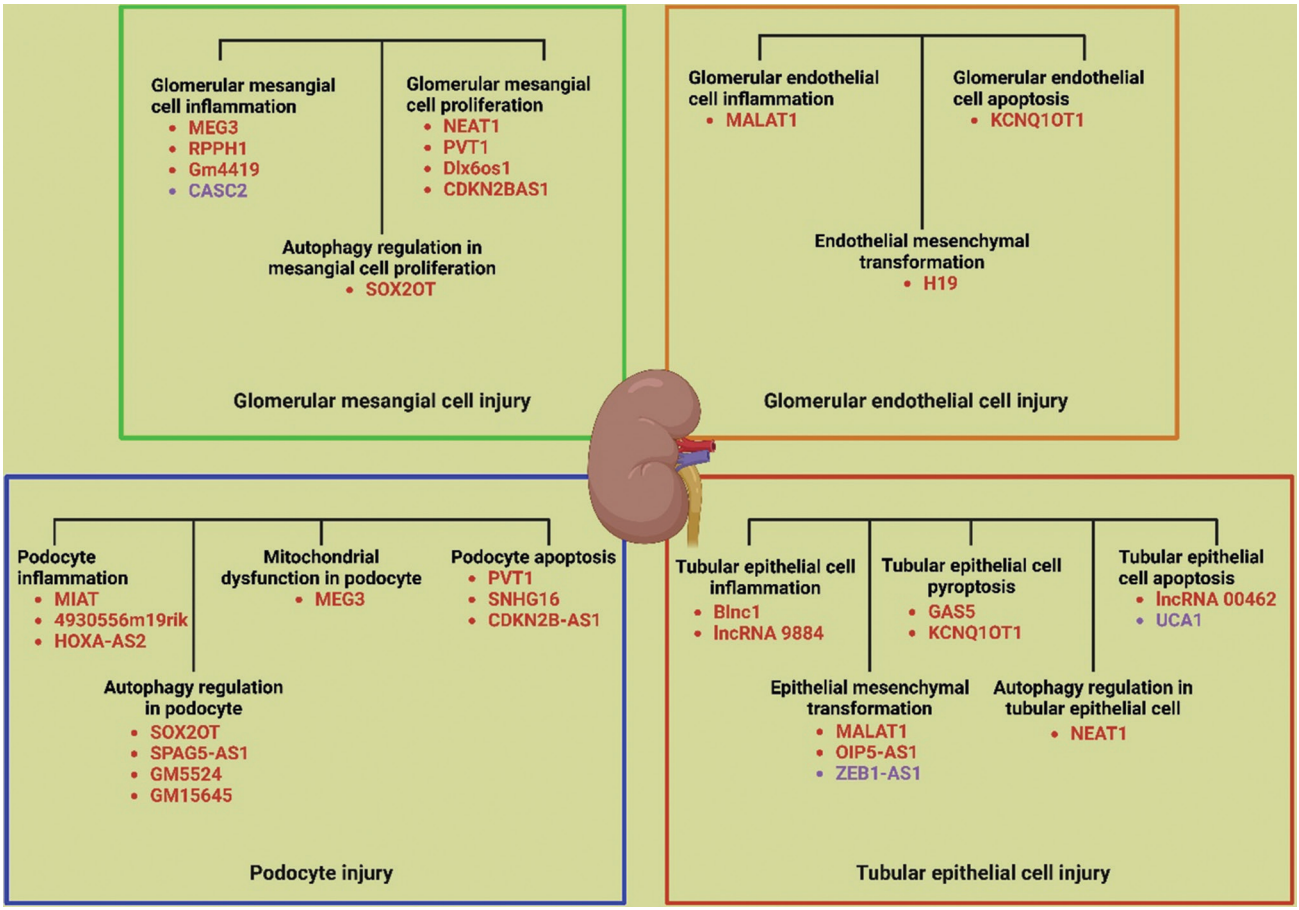

Figure 2. Role of long noncoding RNAs (lncRNAs) in kidney disease among diabetic patients. Dysregulation of upregulated lncRNAs—MALAT1, plasmacytoma variant translocation 1 (LncPVT1), and antisense noncoding mitochondrial RNA-2 (ASncmtRNA-2) and downregulated lncRNAs—CASC2, MIAT, and taurine-upregulated gene 1 (*TUG1*) is crucial in regulating inflammation within the extracellular matrix.
Abbreviations: Blnc1: Brown fat lncRNA 1; CASC2: Cancer susceptibility candidate 2; CDKN2BAS1: Cyclin-dependent kinase inhibitor 2B antisense RNA 1; DIP5-AS1: Antisense RNA; Dlxos1: Distal-less homeobox 1 opposite strand; GAS5: Growth arrest-specific 5; GM5524: Noncoding RNA (mouse); GM15645: Non-coding RNA (mouse); GM4419: Noncoding RNA (mouse); H19: Imprinted maternally expressed transcript; HOXA-AS2: HOXA cluster antisense RNA 2; KCNN4: Potassium calcium-activated channel subfamily N member 4; KCNN1OT1: Potassium calcium-activated channel subfamily N member 1 overlapping transcript 1; lncRNA 9884: Long noncoding RNA 9884; lncRNA 00462: Long noncoding RNA 00462; Long noncoding RNA; MALAT1: Metastasis associated lung adenocarcinoma transcript 1; MEG3: Maternally expressed gene 3; MIAT: Myocardial infarction associated transcript; PVT1: Plasmacytoma variant translocation 1; NEAT1: Nuclear enriched abundant transcript 1; RPPH1: Ribonuclease P RNA component H1; SOX2OT: SOX2 overlapping transcript; SHNG16: Specific RNA sequence; SPAG5-AS1: Sperm associated antigen 5 antisense RNA 1; UCA1: Urothelial cancer-associated 1; ZEB1-AS1: Zinc finger E-box binding homeobox 1 antisense RNA 1; 4930551F19Rik: Noncoding RNA (mouse).

Similarly, other lncRNAs, such as testis development-related gene 1, are dysregulated in DR, promoting microvascular complications. In contrast, lncRNAs like x-inactive specific transcript and lung adenocarcinoma-associated transcript 1 are downregulated, inducing retinal cell apoptosis (Table 3). These findings indicate that the dysregulation of specific lncRNAs plays a key role in DR pathogenesis, suggesting potential therapeutic targets.

4.3. LncRNAs and diabetic cardiomyopathy (DCM)

DCM, significant cardiac complications of DM, is marked by a high risk of heart failure and increased mortality.[64] It is characterized by cardiac dysfunction in patients without coronary artery disease, hypertension, or valvular heart disease.[65,66] Several pathogenic factors contribute to DCM, including oxidative stress, mitochondrial dysfunction, inflammation, impaired calcium handling, angiotensin-converting enzyme activation, and cardiomyocyte apoptosis.[67] The lncRNA H19 plays a key role in DCM, producing a 2.6 kb ncRNA that is polyadenylated, spliced, and mainly localized in the cytoplasm, with some presence in the nucleus. Acute hyperinsulinemia downregulates H19.[68] H19 interacts with the DIRAS3 promoter, recruiting the histone methyltransferase EZH2 to mediate epigenetic

Table 2. Dysregulation of lncRNAs in diabetic nephropathy

LncRNAs	Experimental model	Target site	Functions	References
LncPVT1	Mesangial cells	It has a connection to kidney illness.	PVT1 may affect the growth and development of DN by controlling the buildup of ECM.	40,50
MALAT1	Xenograft mouse model	Extensively expressed in cancers and several mammalian tissues, such as the kidney.	A nuclear buildup of catenin might harm podocytes and eventually result in DN. MALAT1 may facilitate the conversion of catenin in the nucleus by boosting serine/arginine splicing factor 1.	51
ASncmtRNA-2	Human cell lines	This lncRNA is exported to the nucleus from the mitochondria, where it is expressed.	ASncmtRNA-2 can harm human kidneys, and physiological oxidative stress due to the abundance of ROS.	52
TUG1	Rodent retina (newborn retina)	A lncRNA on chromosome 22q12 plays a role in photoreceptor and retinal development in rat retinal cells.	It has been found to have a vital function in the formation of DN besides regulating carcinogenesis in various malignant tumors.	53
MIAT	db/db mice	During a myocardial infarction, detected.	The viability of proximal convoluted tubule cells can be controlled by MIAT by maintaining the appearance of the nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 can functionally and pathologically defend the kidney against diabetes injury.	54
CASC2	clinical study with human patients	In kidney tissues, CASC2 has diagnostic relevance for detecting diabetes complicated by chronic renal failure.	DN with severe kidney failure may be treated and prevented using CASC2 as a target and predictive factor.	55

Abbreviations: CASC2: Cancer susceptibility 2; ECM: Extracellular matrix; DN: Diabetic nephropathy; LncRNAs: Long noncoding RNAs; MALAT1: Metastasis-associated lung adenocarcinoma transcript 1; MIAT: Myocardial infarction-associated transcript; Nrf2: Nuclear factor erythroid 2-related factor 2; PVT1: Plasmacytoma variant translocation 1; ROS: Reactive oxygen species; TUG1: Taurine-upregulated gene 1.

Table 3. lncRNAs involved in diabetic retinopathy

LncRNAs	Experimental model	Dysregulation	Functions	References
MIAT	Diabetic retinas; Endothelial cells cultured in high glucose medium	Upregulated	Encourages swelling, retinal vascular leakage, and the growth of retinal cells	59,60
TDRG1	Human retinal microvascular endothelial cells	Upregulated	Increased microvascular dysfunction	61
XIST	ARPE-19 cells	Downregulated	Encourages retinal cell apoptosis	62
LUADT1	Retinal pigment epithelial cells (RPEpiC, h1RPE7)	Downregulated	Encourages retinal cells to undergo apoptosis	63

Abbreviations: ARPE-19: Adult retinal pigment epithelium 19; LncRNAs: Long noncoding RNAs; LUADT1: Long noncoding RNA associated with diabetic retinopathy; MIAT: Myocardial infarction-associated transcript; RPEpiC: Retinal pigment epithelial cells; h1RPE7: Human retinal pigment epithelial cell line 1 (7); TDRG1: Tumor differentiation regulated gene 1; XIST: X-inactive specific transcript.

repression, inhibiting DIRAS3-induced autophagy, and protecting cardiomyocytes from hyperglycemia's harmful effects.[69]

In addition to its nuclear functions, cytoplasmic H19 acts as a sponge for microRNA-106a (miR-106a) and miR-let7 family members. It also serves as a precursor for miR-675, which regulates genes involved in cell proliferation and growth.[70] Transfecting cardiomyocytes with H19 siRNA reduces miR-675 expression, and a luciferase reporter assay confirmed that miR-675 targets voltage-dependent anion channel 1 (VDAC1). This establishes the H19/miR-675/VDAC1 axis, which helps prevent cardiomyocyte apoptosis under hyperglycemic conditions (Figure 3).[71,72] These findings suggest that targeting the H19/miR-675/VDAC1 axis may offer a novel therapeutic approach for DCM.[12]

5. Biomarkers for DM

Increasing evidence suggests that lncRNAs play a crucial role in disease development. For example, lncRNAs have

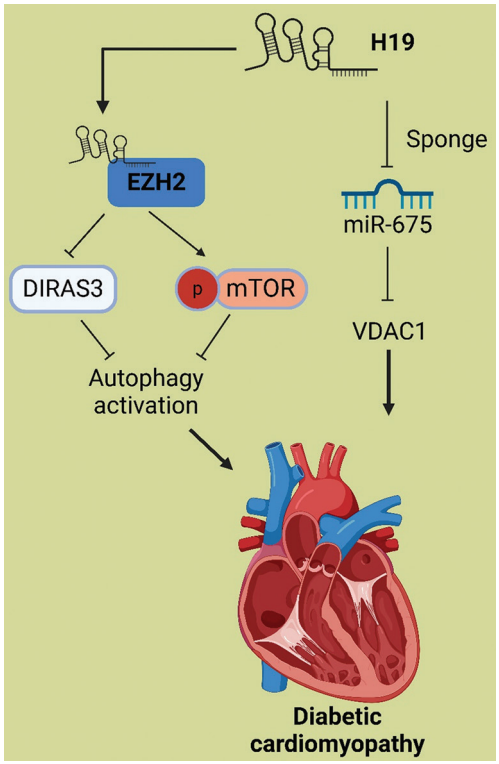

Figure 3. *H19,* the host gene of microRNA-675 (miR-675), reduced hyperglycemia-induced cardiac death by lowering the expression of voltage-dependent anion channel 1 (VDAC1), a target of miR-675. H19 recruited enhancer of zeste homolog 2 (EZH2) and subsequently suppressed DIRAS family GTPase 3 (DIRAS3) expression to prevent autophagy in cardiomyocytes exposed to high glucose levels.
Abbreviation: mTOR: Mammalian target of rapamycin.

been detected in extracellular fluids, such as serum and urine[73] and are emerging as potential biomarkers for diagnosing illnesses, including diabetes.[74,75] The similarity between lncRNAs and exosomes has gained research interest, emphasizing their role in disease mechanisms. For instance, the exosomal secretion of P21-associated ncRNA DNA damage-activated RNA (PANDAR) is highly expressed in hepatoma cell lines, promoting angiogenesis and cell adhesion.[76] A study identified a five-lncRNA signature in plasma exosomes that can diagnose esophageal squamous cell carcinoma.[77] Exosomes also contribute to diabetic disease pathophysiology;[78] exosomal microRNAs secreted by lymphocytes induce pancreatic β-cell death, triggering T1DM.[79] Moreover, mesenchymal stem cell-derived exosomal lncRNA SNHG7 regulates the miR-34a-5p/XBP1 axis, inhibiting tube formation and the endothelial–mesenchymal transition in DR.[80] These findings emphasize the growing clinical relevance of lncRNAs.

Recent studies highlight HOX transcript antisense intergenic RNA (HOTAIR) as a significant predictor, with aberrant expression in various T2DM-related conditions. The human HOTAIR, a 2364-bp lncRNA derived from a 6449-bp gene on chromosome 12q13.13, contains six exons.[81] Its half-life is cell-specific, with about 4 hours in HeLa cells.[82,83] Elevated HOTAIR expression in individuals with DR may serve as a marker to distinguish DR from non-DR.

Moreover, hyperglycemia upregulates HOTAIR expression in human retinal endothelial cells, suggesting its role in DR progression.[84] In DCM, HOTAIR promotes Akt phosphorylation, aiding cardiomyocyte survival.[85] A study in mice on a high-fat diet (HFD) also reported increased HOTAIR expression, reinforcing its strong link to T2DM.[86]

LncRNAs significantly regulate cellular responses and diseases. Growing evidence supports their potential as biomarkers for diagnosing and predicting cancer, viral infections, and autoimmune disorders.[87,89] They are also linked to diabetes, with research highlighting their distinct expression patterns. Wang *et al.* (2013) used qRT-PCR on serum from 96 T2DM patients, finding elevated HOX antisense intergenic RNA as a distinguishing feature and potential noninvasive diagnostic marker and independent predictor of T2DM.[90]

Abnormal expression of lncRNAs has been linked to the pathophysiology of T2DM complications, such as DN and DR.[91] One of the first lncRNAs identified, H19, is essential for β-cell function. Sanchez-Parra *et al.* (2018)[92] showed in rodents that H19 may promote β-cell proliferation by activating Akt and miRNA let-7 Fawzy *et al.* (2020) further found that elevated H19 levels in T2DM patients highlight its potential as a biomarker for insulin resistance.[93]

MEG3, a lncRNA known for cancer suppression, also plays a role in insulin production, secretion, and pancreatic β-cell survival, suggesting it regulates β-cell identity.[94] However, MEG3 overexpression has been associated with hepatic insulin resistance through increased FOXO1 expression and its function as a sponge for miR-214.[95] In addition, MEG3's interaction with miR-185-5p leads to the upregulation of early growth response 2,[96] which inhibits insulin receptor substrate (IRS), further linking MEG3 to insulin resistance.

LncRNAs, such as MALAT1, have been linked to oxidative stress-induced insulin resistance. For example, MALAT1 regulates cell motility and proliferation while suppressing insulin signaling by inhibiting Akt and IRS phosphorylation through c-Jun N-terminal kinase (JNk) activation. Its elevated expression in gestational DM (GDM) highlights its negative regulatory role in insulin signaling.[97] These findings emphasize the critical regulatory roles of

lncRNAs in T2DM and related complications. Moreover, research suggests that modulating lncRNA expression can influence disease pathways.[98] Therapeutic approaches, such as disrupting lncRNA/PRC2 interactions or depleting specific lncRNAs subclasses, have shown potential.[99] For example, Liu *et al*. (2019) found that MALAT1 knockdown in human retinal microvascular endothelial cells reduced microvascular leakage and retinal inflammation in DR.[100,101] Similarly, EPB41L4A-AS1 knockdown has been shown to promote glycolysis and inflammation.[102]

LncRNAs are also involved in key inflammatory pathways, including Janus kinase/signal transducer and activator of transcription, nuclear factor kappa-light-chain-enhancer of activated B cells, and p38 mitogen-activated protein kinase. Notably, elevated MEG8 expression in GDM is associated with kidney damage.[103] Understanding lncRNAs' roles in these pathways may open new therapeutic avenues for diabetic inflammation. Given the impact of lncRNA levels on treatment outcomes, they should be considered in developing personalized therapies. In the future, lncRNAs are expected to play essential roles in diagnosing and treating diabetes-related disorders.

Increased expression of PANDAR in DN may serve as a biomarker to predict outcomes in T2DM.[104] Using exosomes from plasma or serum to detect cell-free nucleic acids offers a novel diagnostic approach for these conditions.[105]

Evidence suggests that lncRNAs regulate gene expression at posttranscriptional, transcriptional, and epigenetic levels, influencing both physiological processes and disease pathways.[106] Aberrant lncRNA expression has been linked to clinical conditions such as psoriasis,[107] cardiovascular disease,[108] diabetes,[109,110] and tumors.[111,112] Studies report that both lncRNAs and miRNAs are dysregulated in insulin resistance.[113] Kornfeld *et al*. (2013) found 66 miRNAs significantly upregulated in the livers of HFD-induced diabetic mice, with 156 miRNAs showing differential expression in Lepr db/db mice homozygous for leptin receptor mutations.[114] These findings underscore the crucial regulatory roles of miRNAs and lncRNAs in T2DM.

LncRNA sequences are conserved across species and show tissue- and cell-specific expression patterns.[115] Advances in next-generation RNA sequencing have enabled the discovery and characterization of numerous lncRNAs. Although 184 lncRNAs have been implicated in human diseases,[116] this represents only a fraction of the potential lncRNA population predicted by bioinformatics tools. Connecting lncRNAs to specific diseases provides insights into complex regulatory systems in human health and offers new therapeutic targets.

Insulin resistance impairs insulin signaling pathways, particularly those involving IRS-1/2, phosphoinositide 3-kinase/Akt, and GLUT4.[117] Emerging evidence suggests that lncRNAs link insulin signaling and sensitivity. Zhu *et al*. (2016) found that MEG3 lncRNA is elevated in the livers of HFD-fed and ob/ob mice, contributing to hepatic insulin resistance by increasing FoxO1 expression.[94] Similarly, Yan *et al*. (2016) reported that MALAT1 lncRNA promotes insulin resistance in hepatocytes by upregulating sterol regulatory element-binding protein-1c in HepG2 cells.[118] Thus, targeting lncRNAs, such as MEG3 and MALAT1 offers potential therapeutic strategies for managing hyperglycemia in T2DM.

LncRNAs have emerged as key regulators in various biological processes, especially in diabetes and its complications. Specific lncRNAs, such as MEG8 and PANDAR, show potential as diagnostic biomarkers for GDM and DN, respectively. However, further research is needed to explore their molecular mechanisms and interactions with other cellular pathways. As advanced RNA sequencing techniques continue to reveal new lncRNAs, they could transform diabetes treatment by serving as molecular markers and therapeutic targets. This research will be essential for developing personalized therapies that effectively manage diabetes-related disorders while reducing side effects and costs.

6. LncRNA therapeutic intervention in DM

LncRNAs have emerged as promising therapeutic targets in DN, offering novel treatment approaches. Preclinical models have investigated various lncRNAs, showing significant potential for intervention. For example, metformin (MET), a widely used drug for managing T2DM, exerts protective effects on the kidneys by activating AMP-activated protein kinase pathways and reducing inflammation and oxidative stress.[119] Notably, MET also modulates lncRNA expression, such as inhibiting H19 in MCs, which mitigates renal damage through the H19/miR-143-3p/TGF-β1 axis.[120]

The antihyperglycemic effects of MET are closely tied to its impact on lncRNA expression. Studies show that MET treatment reverses hyperglycemia-induced increases in H19 and MALAT1 while enhancing GAS5 expression.[121] In addition, clinical findings reveal that patients with T2DM have higher circulating levels of H19, MALAT1, and MEG3 compared to healthy controls, but MET reduces H19 and boosts GAS5 levels. This indicates that MET not only regulates glucose levels but also directly influences lncRNA expression, offering a potential new strategy for treating DN.

Beyond MET, other therapies targeting lncRNAs show promise in DN. Astragalus polysaccharide (APS) has

proven effective in reducing urinary protein and fasting glucose, improving glucose tolerance, and mitigating renal damage in db/db mice. APS promotes autophagy and reduces fibrosis by modulating the lncRNA Gm41268 and its target gene, prolactin receptor, highlighting the potential of lncRNA-targeted therapies for DN complications.[122] Research on nerve injury-specific lncRNA (NIS-lncRNA) suggests that antisense oligonucleotides targeting NIS-lncRNA can relieve pain and suppress inflammatory markers, offering therapeutic benefits not only for DN but also for conditions such as chemotherapy-induced nerve damage.[123]

Despite promising preclinical results, advancing lncRNA-based therapies into clinical practice presents several challenges. A key issue is the quantitative detection of lncRNAs, given their typically low expression levels. Although real-time qPCR is commonly employed, it may lack the sensitivity required for precise measurement. In addition, systemic delivery of lncRNA-targeted therapies risks unintended off-target effects, as lncRNAs regulate multiple pathways simultaneously. Optimizing delivery methods, such as tissue-specific viral or lipid-based vectors, is essential to enhance specificity and minimize side effects.

In summary, lncRNAs offer a promising path for developing targeted therapies in DN, with several preclinical studies yielding encouraging outcomes. However, challenges in detection, delivery, and systemic impact must be resolved before these therapies can transition to clinical practice. Further research, particularly human clinical trials, will be vital for validating the efficacy and safety of lncRNA-based treatments. Overcoming these obstacles is critical to unlocking the potential of lncRNAs as therapeutic tools for managing diabetic complications.

7. Challenges and future directions

As more and more lncRNAs are discovered, the majority of them still have unknown roles and mechanisms of action. Research on lncRNAs related to diabetes is in its early stages. However, recent studies have clearly demonstrated the critical role lncRNAs play in adipogenesis, β-cell activity, and the development of insulin resistance. These discoveries suggest that lncRNAs could serve as viable new therapeutic targets for diabetes and its complications, as well as potential diagnostic indicators. This is undoubtedly a promising new area of diabetes research. lncRNAs are important regulators of various biological processes. Several studies have also highlighted their significance in the emergence of disease, particularly focusing on their role in heart and cardiovascular disease. However, the underlying mechanisms by which lncRNAs influence the inflammation associated with diabetes have not yet been fully explored. This review presents the classification, function, and mechanisms of lncRNAs involved in the regulation of diabetes-related inflammation, along with an introduction to lncRNAs. As demonstrated, lncRNAs can exert their effects by interacting with proteins, RNA, DNA, or combinations thereof. They play a vital role in the initiation and progression of disease. Therefore, this area of research should be pursued further. Future studies should focus on the specific pathophysiology of lncRNAs in diabetic inflammatory disease, with the aim of identifying them as potential therapeutic targets and indicators for diabetes patients, thereby providing new approaches for treating diabetic inflammation through lncRNA-based therapies. We believe that continued research into lncRNAs will yield important discoveries in the future.

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Conflict of interest

Umair Ali Khan Saddozai and Xinying Ji are the Editorial Board Members of this journal but were not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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REVIEW ARTICLE

Orexin in depression: Evidence from basic and clinical research

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Abstract

Orexin – a neuropeptide – is extensively distributed in the central nervous system and is involved in the regulation of diverse physiological functions and behaviors. Orexin is strongly associated with the onset and development of depression. The most important function of orexin is to interact with the transport system, mediating arousal, and energy homeostasis. Hypothalamic-ventral tegmental and hypothalamic-ventral thalamic pathways provide clues for understanding the function of orexin and related disorders, such as sleep disorders, eating disorders, and substance abuse. This article summarizes the basic and clinical studies on the role of the orexin system in regulating and treating depression, including the relationship between the expression level of orexin in specific brain areas and diseases, relationship between adolescent depression and orexin, orexin receptor antagonists as a treatment for depression, and narcolepsy, which is closely associated with depression. Research progress on the role of orexin in depression and recent relevant studies are summarized, providing novel directions for developing depression treatment strategies.

Keywords: Orexin; Depression; Sleep; Major depressive disorder; Neuron

1. Introduction

Orexin – or hypocretin – is a neuropeptide produced by neurons in the perifornical region, lateral hypothalamus (LH), and dorsomedial hypothalamus,[1] as well as by the proteolytic cleavage of a precursor peptide.[2] Orexin comprises two neuropeptides, orexin-A (OXA) and orexin-B (OXB), originating from a shared precursor released by neurons in the hypothalamus.[3] The molecular structures of OXA and OXB comprise 33 and 28 amino acids, respectively (Figure 1). OXA and OXB are formed by cutting the same anterior orexin in neurons on the lateral and posterior hypothalamus and distributed throughout the brain. There are two types of orexin receptors, namely, orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). OXA exhibits equal affinity for both receptors, whereas OXB preferentially binds to OX2R. Orexin nerve fibers project to the spinal cord and wide

areas of the brain. These projections are closely connected with neurons in the locus coeruleus, limbic system, ventral dorsal opercular area, and ventrolateral preoptic nucleus, among others (Figure 2). These interactions exert various physiological functions. Orexin concentrations increase in certain pathological conditions, such as posttraumatic stress disorder (PTSD), and decrease in other conditions, such as memory deficits.[4]

The orexin system is an important regulator for several neural functions and a valuable drug target.[5] Furthermore, orexin neuropeptides have received increasing attention in neuroscience research and are applied in numerous fields, including reward and motivation, addiction mechanisms, sleep/wake regulation, appetite regulation, and depression and anxiety research.[6] Inhibition of orexin neurons and a decrease in orexin levels can cause depression; by affecting the sleep–wake cycle, these changes can cause narcolepsy and result in learning and memory deficits.[7] Low activity of the orexin system is also associated with paroxysmal sleeping sickness.[8]

The role of orexin in depression potentially stems from its pivotal role in regulating arousal and mood through complex neurophysiological interactions.[9] By regulating the neurotransmitter systems, orexin contributes to the neurobiological processes underlying mood disorders, which targets orexin as a potential factor for novel

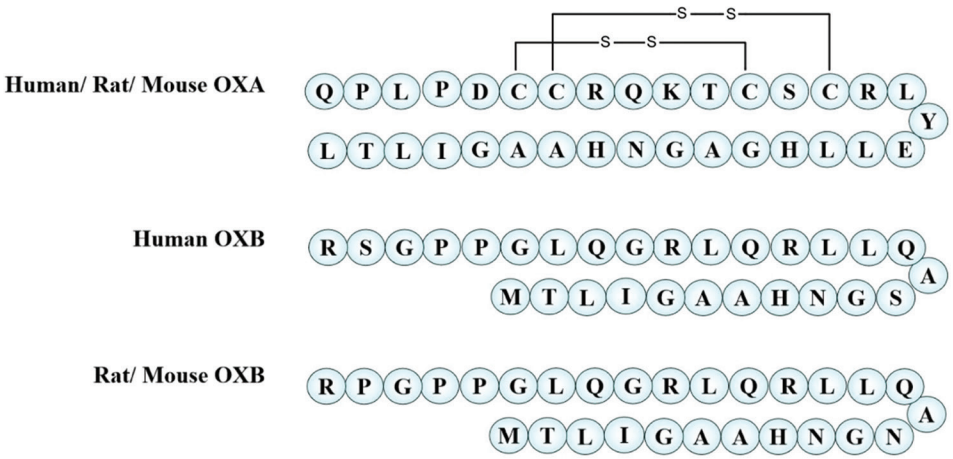

Figure 1. Amino acid sequence of OXA and OXB in different species. Orexin is distributed in humans, rats, and mice. Among the three species, the amino acid sequence of OXA was consistent, whereas that of OXB was different. Figure created by author.
Abbreviations: OXA, Orexin-A; OXB, Orexin-B.

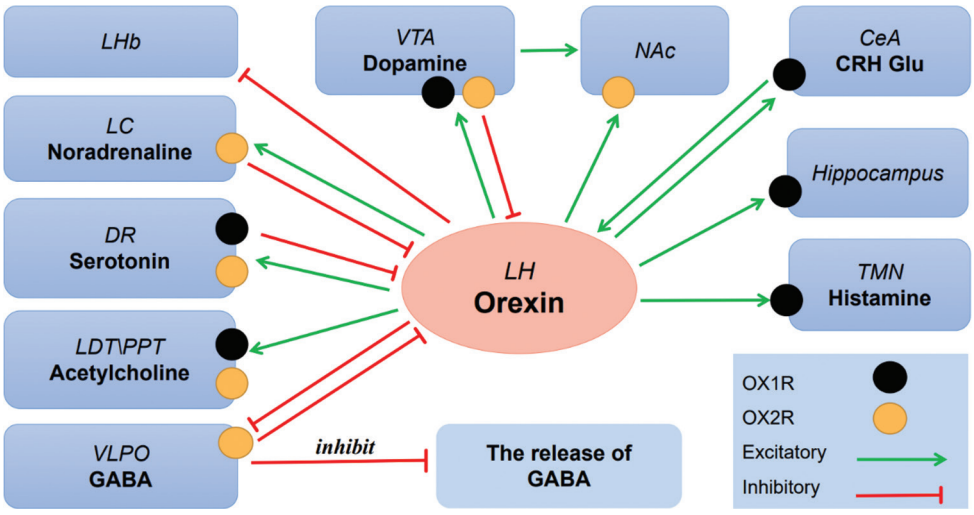

Figure 2. Schematic of the role of the orexin system in the central nervous system
Abbreviations: CeA: Central amygdala; CRH: Corticotropin-releasing hormone; DR: dorsal raphe; GABA: Gamma-aminobutyric acid; Glu: Glutamic acid; LC: Locus coeruleus; LDT: Lateral–dorsal tegmental nucleus; LH: Lateral hypothalamus; LHb: Lateral habenular nucleus; NAc: Nucleus accumbens; PPT: Pedunculopontine nucleus; TMN: Tuberomammillary nucleus; VLPO: Ventrolateral preoptic area; VTA: Ventral tegmental area.

antidepressant therapies. The cAMP-response element-binding protein (CREB) is a transcription factor binding to specific DNA sequences and regulating gene expression. The CREB signaling pathway has been identified as a key intracellular mechanism influenced by orexin, which may clarify its impact on energy homeostasis and the sleep–wake cycle. Orexin can affect the CREB activity by activating intracellular signaling pathways. In an inactive state, CREB may be present in the cytoplasm; however, on stimulation by specific signals, such as activation by the cAMP/PKA (protein kinase A) pathway, CREB is phosphorylated and translocated to the nucleus,[10] where it plays a remarkable role in regulating energy homeostasis and wakefulness (Figure 3). This indicates that orexin is essential for maintaining a state of arousal.[11] Orexin neurons regulate various physiological activities through interactions with monoaminergic, dynorphin, and serotonergic neurons (Figure 4).[12] Transcripts controlled by orexin control energy metabolism and sleep and are associated with depression.

Orexin neurons critically modulate arousal and reward processing by interacting with key neurotransmitter systems, remarkably the dopamine and GABAergic pathways.[13] Specifically, the influence of orexin on the CREB pathway emphasizes its potential to regulate energy balance and arousal at the cellular level. The activation of orexin receptors modulates synaptic plasticity, influencing mood-related behaviors through direct interactions with the hypothalamic-pituitary-adrenal (HPA) axis (Figure 5) and other critical stress-response circuits;[14,15] this complex interplay may underlie the observed decrease in orexin levels in depressive states, thereby linking orexin dysregulation to depression pathophysiology.

This review article aims to provide novel insights into the use of orexin in the treating and preventing depression. This article discusses orexin expression levels,

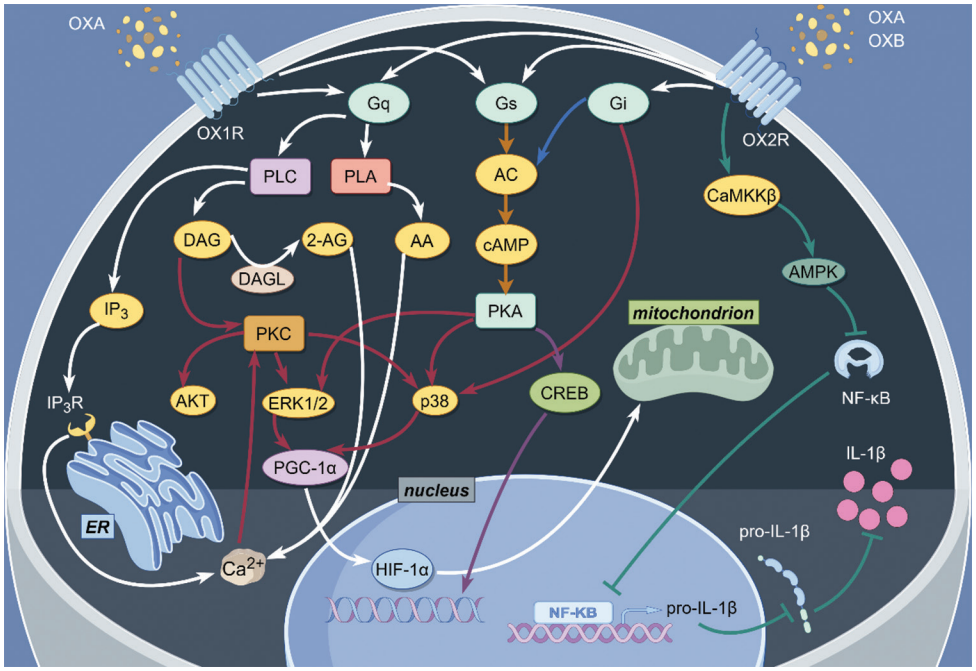

Figure 3. Orexin signaling pathway was observed in neuronal cells. The PLC pathway is generally activated by Gq protein activation, which further activates PLC, DAG/IP3, the PLC-DAGL enzyme cascade and PKC, and finally induces Ca^{2+} elevation. The PLA pathway is associated with Ca^{2+} elevation, which further regulates the activity of this pathway. The AC pathway is regulated by Gs and Gi proteins, thereby exerting opposite effects. cAMP and PKA are activated after AC activation. ERK1/2 and p38 are activated in response to orexin, further activating PGC-1α, the primary inducer of adenosine triphosphate (ATP) production. PGC-1α regulates HIF-1α expression, and elevated HIF-1α causes changes in gene expression, mediating energy homeostasis. The activation of energy-sensing AMPK in the central nervous system (CNS) is closely related to orexin and can inhibit NF-κB activation, thereby preventing NF-κB from initiating the expression of inflammatory genes, improving the inflammatory state, and affecting depression. (Created using Figdraw.com).

Abbreviations: AA: Arachidonic acid; AC: Adenylyl cyclases; AKT: Ak strain transforming kinase; AMPK: Adenosine monophosphate-activated protein kinase; CaMKKβ: Calcium/calmodulin-dependent protein kinase; cAMP: Cyclic adenosine monophosphate; CREB: cAMP-response element-binding protein; DAG: Diacylglycerol; DAGL: Diacylglycerol lipase; ERK1/2: Extracellular-signal-regulated kinase 1/2; HIF-1α: Hypoxia-inducible factor-1 alpha; IP3: Inositol trisphosphate; IP3R: Inositol trisphosphate receptor; NF-κB: Nuclear factor kappa-B; PGC-1α: Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha; PKA: Protein kinase A; PKC: Protein kinase C; PLA: Phospholipase A; PLC: Phospholipase C; P38: Mitogen-associated protein kinase 38; 2-AG: 2-Arachidonoylglycerol.

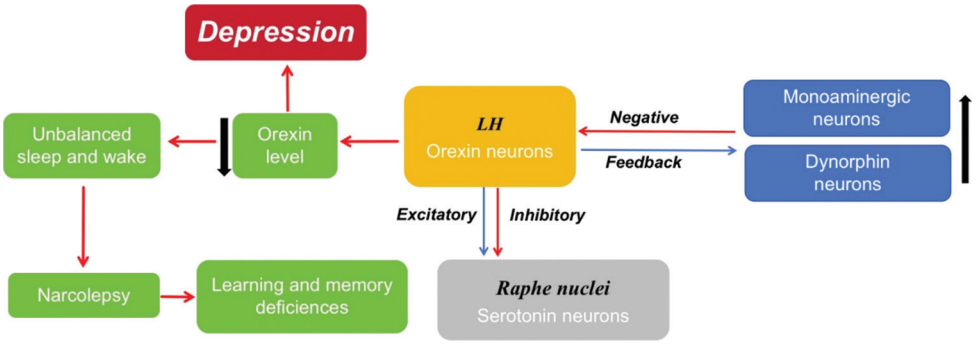

Figure 4. Subsequent effects of orexin neuron inhibition. The straight lines connecting orexin neurons and serotonin neurons indicate that low concentrations of orexin exert a direct excitatory effect on serotonin neurons, whereas high concentrations indirectly inhibit them. Figure created by author.
Abbreviation: LH: Lateral hypothalamus.

receptor function, animal models, and clinical studies. Furthermore, it illustrates the potential mechanisms of the orexin system in the onset and development of depression, such as its effects on sleep–wake cycle, reward processing, stress response, and energy balance. Finally, the review evaluates the potential of orexin receptor antagonists as a treatment for depression.

2. *In vitro* studies on the role of orexin in depression

Disturbances in orexin production can cause sleep disorders and depression and affect motor activity. Nevertheless, to date, specific changes caused by the orexin system are not reported. In a previous study, corticosterone injections were used to examine orexin neurons and corresponding behavioral changes in mice with stress-induced depression; reportedly, significant levels of external stress-induced depression may be functionally associated with increased orexin neuronal activation.[16]

The hypothalamic neuropeptide orexin is related to the pathophysiology and accumulation of mental diseases. Empirical data indicate a possible association between depression and orexin. Nonetheless, the exact role of orexin in depression remains unknown, particularly regarding the underlying neurological system and mechanism. Ji *et al.* found that a direct projection exists from the ventral pallidum; the structure is becoming increasingly important due to its role in reward processing, depression, and stress reactions. These data demonstrate that orexin inhibits depressive-like behaviors in rats by directly activating GABAergic neurons in the ventral pallidum.[17]

Chronic life stressors can increase the risk of depression, often causing imbalances in the body. Prolonged stress causes changes in neuronal functioning in particular brain areas that control sociability and mood-related behaviors. The LH sends orexin fibers to the central amygdala, which then expresses OX1R (Figure 2). The central amygdala – the primary output of the amygdala – is vital for processing emotions.[18] The KCNQ3 gene encodes a voltage-gated potassium channel subunit expressed in the brain. The PCDH10 gene – protocadherin 10 – is a member of the protocadherin subfamily within the cadherin superfamily. KCNQ3 and PCDH10 genes play key roles in nervous system function, and their mutations or abnormal expression may be associated with various neurological disorders. Changes in the expression of KCNQ3 and PCDH10 genes can disrupt the normal control of the effect of the amygdala on orexinergic neurons by affecting neuronal excitability, synaptic transmission, neuropeptide expression, neurodevelopment, and neural circuits, potentially overactivating the hypothalamic orexin system.[19] Persistent high levels of orexin and melanin-concentrating hormone (MCH) in the basolateral amygdala (BLA) caused by stress, as well as the activation of orexin receptors or MCH receptors in the BLA, have been linked to disruptions in social interactions and mood-related behaviors. The occurrence of high levels of MCH is related to the activation of the sympathetic nervous system as a result of stress, producing norepinephrine and results in the excessive proliferation of melanocyte stem cells.[20] It may also relate to the activation of the HPA axis (Figure 5).[21] A study of neural targets influenced by BLA neurons containing MCH or orexin receptors suggested that BLA neurons play a role in social and emotional behaviors modulated by the orexin and MCH receptor systems.[22]

The relationship between depression and MCH-ergic system or hunger remains unclear. Thus, the impact of extended treatment with selective serotonin reuptake inhibitors on orexin and MCH neuronal activity in mice subjected to prolonged mild stress was investigated; this was achieved through dual immunohistochemical labeling of orexin- or MCH-containing neurons and c-Fos protein

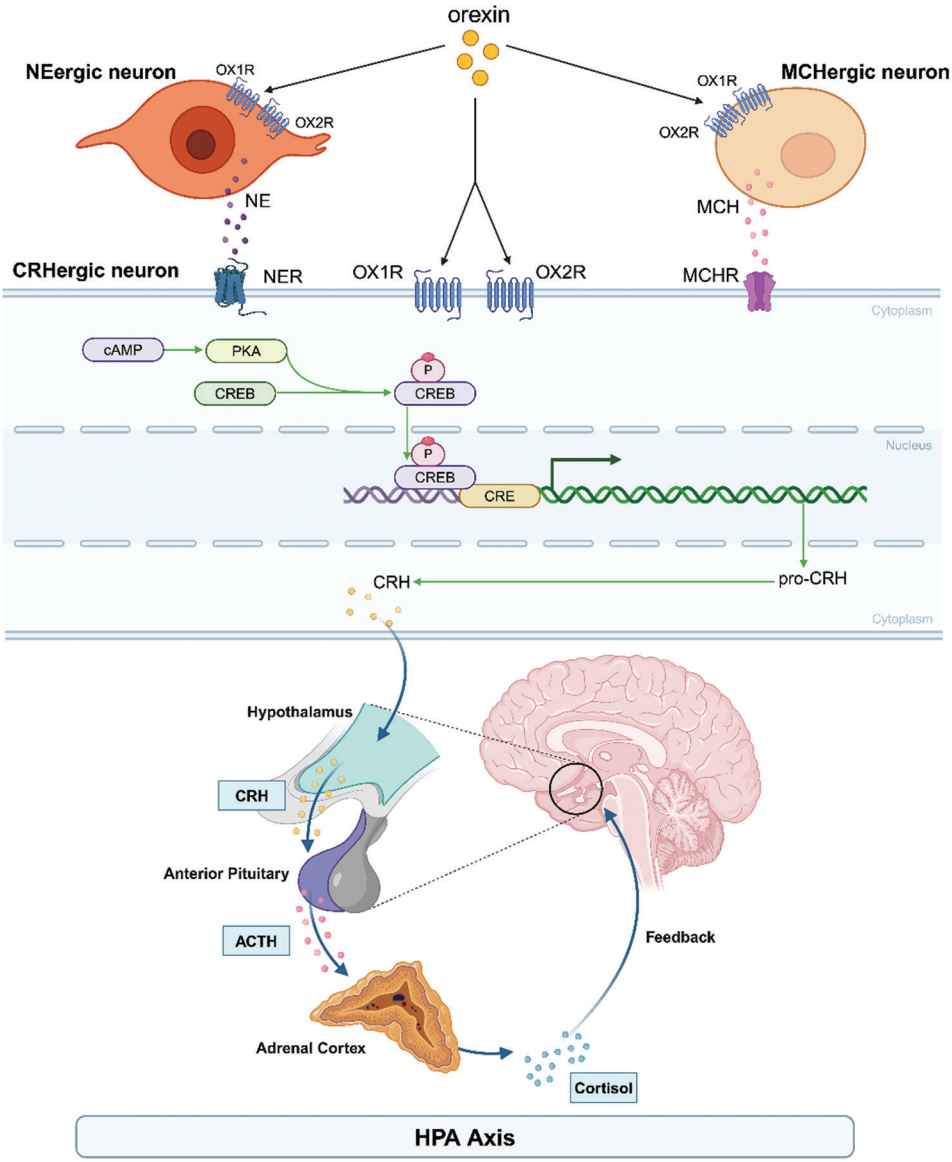

Figure 5. Relationship between orexin and the HPA axis. Orexin can directly bind to the orexin receptor on CRHergic neurons and can influence CRHergic neurons by binding to the orexin receptors on NEergic or MCH-ergic neurons. When activated by orexin, the level of cAMP in CRHergic neurons increases, which activates PKA, phosphorylates, and CREB. p-CREB promotes the transcription of the CRH gene, stimulating CRH synthesis and release. CRH is a key regulator of the HPA axis and can stimulate the anterior pituitary to release ACTH, further facilitating the release of glucocorticoids (such as cortisol) in the adrenal cortex. Cortisol – a major hormone in stress response – could affect emotional regulation in the brain. Chronic or excessive stress may cause dysregulation of the HPA axis, which is associated with the onset of depression. (Created using biorender.com).
Abbreviations: ACTH: Adrenocorticotropic hormone; cAMP: Cyclic adenosine monophosphate; CRE: cAMP-response element; CREB: cAMP-response element-binding protein; CRH: Corticotropin-releasing hormone; HPA: Hypothalamic-pituitary-adrenal; MCH: Melanin-concentrating hormone; MCHR: Melanin-concentrating hormone receptor; NE: Norepinephrine; NER: Norepinephrine receptor; PKA: Protein kinase A.

(a molecular marker of neuronal activity). The findings suggested that orexin neurons in the dorsomedial and perifornical hypothalamic regions, as well as the MCH-ergic systems, play a role in the onset of depressive disorders.[23] MCH and orexin neurons support each other in numerous physiological processes, including energy balance and the circadian rhythm of sleep and wakefulness. *In vitro* electrophysiological studies investigating these cells often use postweaning rodents.[24,25] Nevertheless, the functional maturity these neurons at this time remains unclear. Studies on the electrical characteristics of MCH neurons and orexin in brain slices obtained from postweaning rats suggested that orexin neurons, after receiving sufficient stimulation before puberty, can generate action potentials

through the opening and closing of ion channels. These action potentials are then transmitted along the axon to the presynaptic terminal, in which they transmit the electrical signal to the next neuron through the release of neurotransmitters. Meanwhile, MCH neurons continue to develop until late puberty.[26] These data provide some guidance for judging the onset time of depression.

Orexin-producing hypothalamic neurons communicate their predominantly neuroexcitatory signals throughout the brain through extensive axonal projections.[27] A major noradrenergic nucleus of the brain is the locus coeruleus, to which the LH sends abundant orexin connections (Figure 2).[28] Neurons in the LH that respond to glucose levels produce OXA and OXB and project their axons to the hippocampus (Figure 2), where OX1R, primarily sensitive to OXA, is predominantly expressed. The behavior of rats was evaluated using the Morris water maze test, and the impact of OXA on synaptic transmission at the Schaffer collateral/commissural-CA1 pathway in hippocampal slices was investigated, focusing on long-term depression and potentiation. The obtained results emphasized the adverse effects of OXA on spatial cognition, which can be attributed to the suppression of long-term potentiation in the Schaffer collateral-CA1 hippocampal synapses.[29]

Corticotropin-releasing hormone (CRH) and orexin are involved in modulating excitatory synaptic transmission in the ventral cover region.[30] The clinical manifestations of depression often include disrupted sleep patterns. CRH may influence sleep. Neurons from the arcuate nucleus that secrete the neuropeptide Y/agouti-related peptide and anorexigenic proopiomelanocortin/cocaine- and amphetamine-regulated transcript peptides project to the LH, regulating orexin-containing neurons, which increase food intake, and they also project to the paraventricular nucleus, regulating CRH neurons to decrease feeding.[31] Considering the anatomical and neurophysiological interactions between orexin and the CRH system, these two neuropeptides hypothetically interact with each other. To explore this hypothesis, the impact of dysfunction in the CRH receptor system on the wake-promoting effects of exogenous orexin was investigated using two different CRH receptor knockout models; notably, the possible action of CRH did not affect the wake-promoting effect of orexin.[32] The epidermal growth factor receptor (EGFR) controls neural activity in vertebrates and the sleep–wake cycle. Hypothetically, EGFR signaling mediates the orexin system activity during the onset of sleep.[33]

Endogenous adenosine, produced by cells within the body, promotes sleep. When ATP is released from inside the cell into the extracellular space and destroyed by ecto-ATPases, endogenous adenosine is generated.[34] It plays a role in controlling neuronal excitability and synaptic communication in the central nervous system (CNS). A study investigating the role of endogenous adenosine in controlling excitatory glutamatergic synaptic transmission to orexin neurons indicated that endogenous adenosine within the hypothalamus is released into the extracellular space in an activity-dependent manner, preventing the long-term potentiation of orexin neurons and basal excitatory synaptic transmission through adenosine A1 receptors.[35] Caffeine, a nonselective adenosine receptor antagonist, exerts stimulating effects on arousal and sympathetic activity. In sleep-deprived mice, the administration of caffeine resulted in a remarkable increase in spontaneous activity. In particular, the wake-promoting effects of caffeine in sleep-deprived mice were reversed by an orexin receptor antagonist, indicating that orexin is essential in mediating the pharmacological effects of caffeine.[36]

Using whole-cell patch-clamp recordings on acute sections of the dorsal raphe nucleus (DRN), OXB exerts a suppressive effect on glutamate-induced synaptic currents within DRN serotonin neurons.[37] In acute mouse brain slices, glycine – a neurotransmitter known for its involvement in the brainstem and spinal cord – induces dose-dependent postsynaptic Cl^- currents in orexin cells. Pharmacological analysis of glycine receptor responses revealed that although orexin neurons in the early postnatal period possess the α2-subunit, mature orexin neurons possess α/β-heteromeric glycine receptors, implying that the two pools of glycine receptors control the activity of growing orexin cells.[38]

For obesity or short-term food deprivation, OXA neurons exhibit increased activity levels, resulting in hyperarousal and an increased drive to seek food.[39] Although brief exposure to a high-fat diet can cause synaptic plasticity in the mesolimbic pathway, whether orexin neurons are affected by this modification remains unclear. Reportedly, the intake of appetizing high-fat diets can trigger long-lasting synaptic depression in excitatory connections targeting orexin neurons; this finding suggests the presence of a homeostatic mechanism aimed at preventing the excessive activation of these neurons and reducing the intake of high-fat diets.[40] A brief exposure to a high-fat diet may increase the levels of certain excitatory neurotransmitters in the brain, resulting in prolonged inhibition of orexin neurons. The neuropeptide orexin, synthesized by neurons located in the LH, is associated with obesity and anxiety-related depression. The protein delta-like homology 1 (DLK1), expressed by every orexin neuron, may aid in the control of anxiety-related depression and energy balance. DLK1 was concurrently expressed by all rat orexin neurons, which is consistent

with earlier findings.[41] DLK1-null mice exhibited reduced anxiety and depression levels, as indicated by the results of the forced swim test (FST) and elevated plus maze, along with increased locomotor activity, compared with those in the control mice. These results indicate that DLK1 plays a predominant role in depressive behavior through DLK1-expressing orexin neurons.[41]

The awakening and maintenance of the waking state depend on the hypothalamus orexin system. The LH is integral to brain function and involved in substance abuse. Neuroendocrine information pertaining to natural rewards is consolidated by orexin neurons within the LH. Drugs of abuse may exert their effects concurrently at the LH and ventral tegmental area. The presence of rewards or cues associated with them causes an elevation in c-Fos expression and phospho-CREB levels within orexin neurons. OXA activates a $G_{q/11}$ protein coupled–receptor mediated phospholipase C (PLC)-diacylglycerol lipase (DAGL) enzymatic pathway in dopaminergic neurons in the ventral tegmental area, resulting in the production of 2-arachidonoylglycerol (2-AG) – an endocannabinoid (Figure 3). The 2-AG then inhibits GABAergic neurons by binding to cannabinoid receptor 1, which reduces GABA release.[42] The process of glutamate binding to AMPA and NMDA receptors might cause the disinhibition of dopaminergic neurons in the ventral tegmental area,[43] where glutamate can enter dopaminergic neurons through NMDA receptors and promote the activation of PLC, further affecting the function of NMDA receptors[44] (Figure 6). This process subsequently allows orexin to regulate reward and incentive behaviors.

The extensive projection of orexin neurons, ability to sense an animal's internal state, and high plasticity of signals pertaining to natural rewards and drug abuse may be the primary factors for increased drug-seeking behaviour.[45] Research indicates that mechanical stimulation (MS) of the ulnar nerve effectively reduces cocaine addiction behaviors – an effect potentially associated with the orexinergic input from the LH to the lateral habenular nucleus (LHb).[46] The axons of orexinergic neurons in the LH can directly extend and form synaptic connections, establishing direct synaptic contacts with neurons in the LHb.[47] Systemically administering OX2R antagonists inhibited the activation of LHb neurons by MS, and MS targeting the ulnar nerve engaged an orexin LH-LHb pathway to dampen cocaine-induced psychomotor responses.[46] Hypothalamic neurons are inhibited by opioids, which can reduce cognitive alertness and result in inhibiting orexin awakening system. Exogenous opioids inhibit the orexin system through direct actions on the cell body, resulting in the development of depression.[48]

Although current *in vitro* studies have investigated the relationship between orexin and depression, researchers have used several acute stress models, which can hamper the chronic and complex nature of human depression. Moreover, there is limited research on the translational potential, particularly how *in vitro* findings might inform the development of specific orexin receptor modulators as therapeutic interventions for depression. Future research should consider the chronic modulation of orexin activity in depression models that mimic human pathophysiology for elucidating the relevance of these findings.

3. Orexin in animal models of depression

Recent studies have emphasized the role of orexin system in regulating stress response and emotional behaviour,[49] making orexin an important hotspot in the animal models of depression.

Increased orexin signaling is associated with depression-like states, and pharmacological interventions targeting the orexin system can yield antidepressant-like effects. For instance, blocking orexin receptors in mice subjected to unpredictable chronic mild stress resulted in substantial behavioral improvements, suggesting that orexin antagonism counteracts stress-induced alterations in mood and behaviour;[50] this finding is consistent with the notion that orexin signaling contributes to motivational states, which can become dysregulated in depression.[51]

Motivation and stress are key factors in depression, with orexin signaling systems modulating motivation and stress responses.[52] A previous study investigated the correlation between orexin and its receptors in brain regions related to depression by examining immobility in mice during FST.[53] The analysis focused on the mRNA expression of orexin and its receptor concerning FST immobility. Results indicated an inverse correlation between depressive behavior severity and hippocampal orexin expression, along with elevated mRNA levels of orexin and its receptor in the amygdala. This unique relationship between the amygdala and hippocampus represents a neurobiological motif appearing in depression, that is, the amygdala grows in size and function, whereas the hippocampus shrinks.[53]

After stressful life experiences, there is an increased risk of developing psychiatric illnesses, such as depression, anxiety, and posttraumatic syndrome, throughout adolescence, a sensitive and crucial time for brain development. Individuals with these conditions exhibit alterations in orexin levels.[54] However, the exact function of the orexin system in modulating these emotional manifestations remains uncertain. The medial prefrontal cortex plays a role in emotional as well as cognitive processing. Behavioral changes related to PTSD in

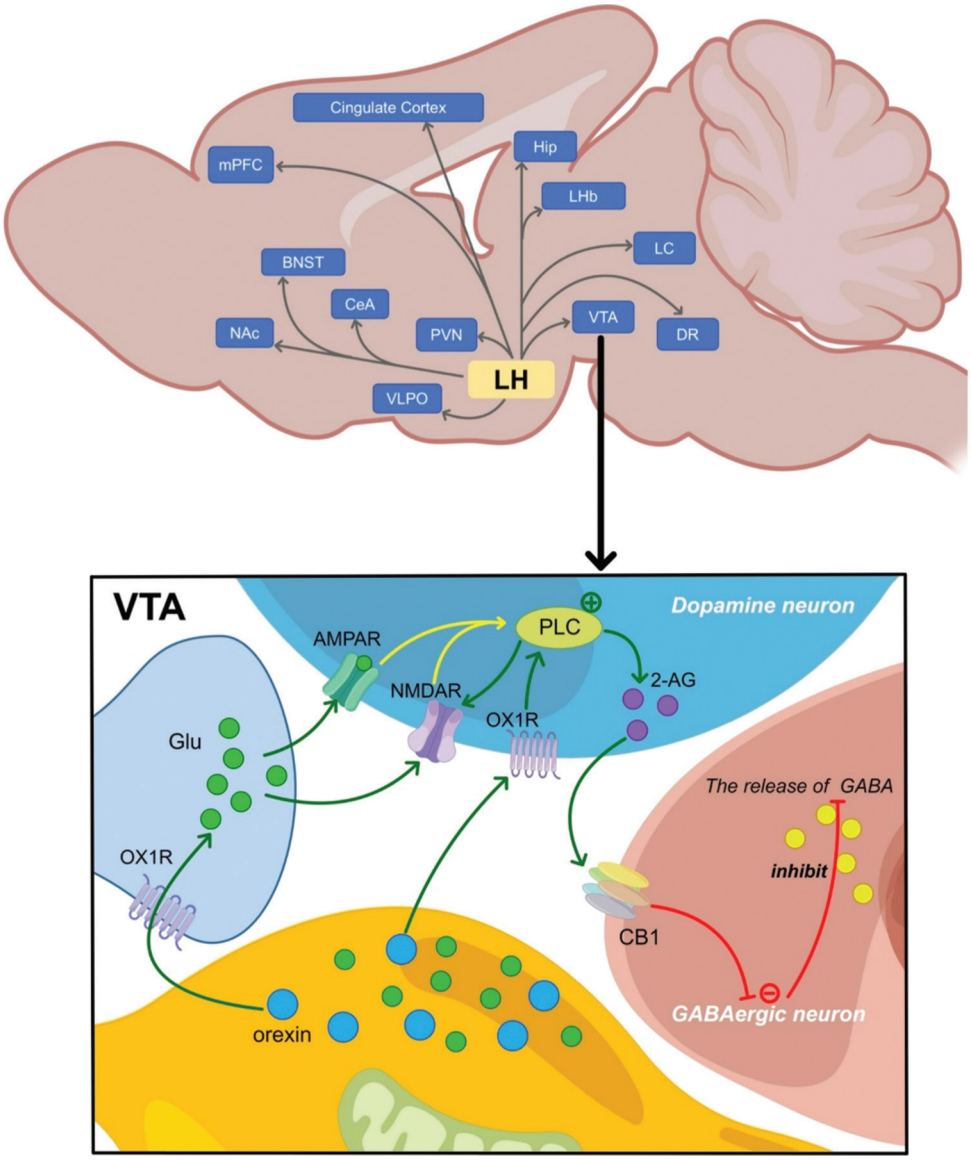

Figure 6. Diagram of pathways involved in orexin signaling. Acting on presynaptic OX1R in the VTA, orexin can stimulate the release of glutamate onto dopamine neurons to regulate complex behaviors and physiological processes related to arousal, motivation, reward, food intake, emotion, and cognition. OX1R activation in dopamine neurons can recruit PLC, inhibiting the release of GABA from interneurons. (Created using Figdraw.com).
Abbreviations: AMPAR: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; BNST: Bed nucleus of the stria terminalis; CB1: Cannabinoid receptor 1; CeA: Central nucleus of the amygdala; DR: Dorsal raphe; Hip: Hippocampus; KOP: κ-opioid receptors; LC: Locus coeruleus; LH: Lateral hypothalamus; LHb: Lateral habenula nucleus; MOP: μ-opioid receptors; mPFC: Medial prefrontal cortex; NAc: Nucleus accumbens; NMDAR: N-methyl-d-aspartic acid receptor; PLC: Phospholipase C; PVN: Hypothalamic paraventricular nucleus; VLPO: Ventrolateral preoptic area; VTA: Ventral tegmental area; 2-AG: 2-Arachidonoylglycerol.

male rats induced by single prolonged stress (SPS) were observed through elevated cross-maze, sucrose water preference, and wilderness experiments. Rats exposed to SPS exhibited increased anxiety levels, reduced exploration activities, and a lack of pleasure. Moreover, there was an increased OX1R expression in the medial prefrontal cortex and a decreased OXA expression in the hypothalamus as a consequence of SPS. Lateral ventricular injection of OXA diminished the behavioral alterations in SPS-exposed rats and partially recovered the increased OX1R level in the medial prefrontal cortex; these findings demonstrate that PTSD symptoms associated with depression and anxiety are influenced by the orexin system.[55,56]

A link between depression and the orexin system may exist, and the pathological regulation of depression may relate to orexin.[57] Acquired helplessness (AH)

describes powerlessness that originates from an individual experiencing events or situations beyond their control. Reportedly, an animal model of AH depression was used to distinguish rats with AH behavior (AH rats) from those without AH behavior (No-AH rats).[58] The number of orexin-containing neurons in the hypothalamus was compared among AH, No-AH, and control rats. The concentrations of OXA/OXB peptide, OX1R, and OX2R in the brain regions associated with severe depression and those of OXA and corticosterone in the serum were quantified and compared.[58] Higher serum OXA concentrations were observed in AH and No-AH rats than in the control rats. When AH and No-AH rats were compared, the No-AH rats showed higher brain OXA levels and more OXA neurons and activity. The number of OXB neurons and their activity were higher in AH rats. In AH and No-AH rats, orexin receptors and peptides exhibited distinct patterns in the brain regions pertaining to severe depression. OXA and OXB play different roles in AH behavior, and OXA neuron activity may foster resilient behavior under stress.[58]

The pathophysiology of depression is related to reduced OXA levels in the CNS, and exogenous treatment of OXA can improve antidepressant effects. However, the processes that underlie these effects remain uncertain. A recent report indicates that the impact of OXA was related to the activation of tyrosine receptor kinase B (TrkB) and OX1R in the ventromedial prefrontal cortex (vmPFC), a neurobiological hub associated with depression.[59] To analyze the involvement of TrkB and OX1R in the behavioral effects exerted by OXA, rats were injected with 10, 50, and 100 pmol/0.2 μL of OXA into the vmPFC for the FST and open field test. OXA signaling in the vmPFC induced OX1R and Trk receptor-dependent antidepressive-like effects in the FST.[59]

The mechanisms and key locations of the integration of OXA signaling and photocycle are poorly defined. One study explored the effects of OXA on melatonin concentrations in the cerebrospinal fluid and plasma at night in sheep, wherein the expression of OX1R in the pineal gland was examined during short and long days.[60] The results implied that day length and nonoptical cues through hypothalamic OXA regulate the endocrine activity of the pineal gland in sheep; this finding has considerable ramifications for our comprehension of the circadian clock functioning and processes by which seasonal animals adjust to their environment. Because OXA and melatonin play a role in regulating the sleep–wake system, the findings have applications in disorders of the human circadian rhythm, including jet lag, sleeplessness, and seasonal depression.

OXA and OXB are involved in controlling various behaviors and homeostasis aspects, including energy homeostasis and sleep–wake cycles. The autonomic nerve system, reward system, and sleep–wake state are closely, anatomically, and functionally related to the orexin system. The autonomic nervous system, reward system, and emotion regulation may be influenced by the physiological functions of OX1R, according to pharmacological studies using selective antagonists. In one study, mice were tested through a comprehensive suite of behavioral tests to screen for additional functions of OX1R; the findings indicated that besides its involvement in regulating mood and anxiety, OX1R influences social behavior and sensorimotor gating.[61]

Moreover, the relationship between orexin and the HPA axis is particularly interesting. Dysregulation of the HPA axis is a hallmark of depression, and orexin influences the HPA axis activity (Figure 5). In animal models, chronic treatment with orexin receptor antagonists alleviated depressive-like behaviors and restored the normal HPA axis function, indicating a potential therapeutic pathway for managing depression.[23,62]

Food intake is regulated by the CNS through detection, integration, and reaction to numerous internal and external cues. Eating behavior is significantly affected by hedonic and reward-related factors.[63] Type 2 diabetes mellitus and insulin resistance are related to depression; however, the molecular processes underlying this clinical relationship remain unclear. Orexin controls glucose homeostasis and energy levels, which has been associated with endogenous antidepressant processes. Mice with chronic social failure anxiety were administered an orexin-deficient diet, and their social behavior and glucose metabolism were examined to assess whether orexin is involved in social behavior and metabolic regulation. Calorie restriction potently activates orexin neurons and may stop the persistence of behaviors resembling depression, thereby improving impaired glucose metabolism after prolonged stress. Therefore, the orexin system is crucial for preventing the worsening of the relationship between depression and type 2 diabetes.[64]

Eating disorders are a common component of several psychiatric illnesses; however, there is limited understanding of the neurobiology underlying the behavioral modifications induced by temporary calorie restriction. At present, rodent forms of depression considerable respond to a 10-day calorie restriction, and this impact is orexin-dependent. After calorie restriction, the wild-type mice exhibited longer immobility latency and lower complete immobility in FST. Calorie restriction corrected the behavioral abnormalities in wild-type mice but not in orexin-mutant animals in a social defeat paradigm of chronic stress. Furthermore, chronic social

failure stress causes a long-term decrease in pre–pro orexin mRNA expression at the orexin gene promoter through epigenetic modification, whereas calorie restriction improves orexin cell activation after social defeat. To summarize, orexin is crucial in regulating the decrease in depression-like symptoms caused by calorie restriction.[65]

Decreased orexin function is a potential reason why people at a high risk of developing cardiovascular diseases are prone to depression.[66] Although the exact cause of depression in patients with cardiovascular diseases such as obstructive sleep apnea (OSA) and sudden infant death syndrome (SIDS) remains unknown, patients with OSA and SIDS experience recurrent episodes of respiratory arrest and/or insufficient breathing, causing hypoxia/hypercapnia (H/H).[67,68] Those studies investigated the responses of fluorescently identified rat orexin neurons in the LH to acute H/H to test whether and how these neurons alter their activity and function during this challenge; the results suggested that the inhibition of orexin neurons is related to the H/H-induced reduction of glutamatergic neurotransmission within orexin neurons.

Overall, the orexin system has demonstrated remarkable regulatory functions across different animal models of depression, suggesting that its control in depression is universal. Although there is a close relationship between orexin and mood disorders, current research primarily depends on animal models, which to some extent limits translational research related to clinical practice. Moreover, most existing studies focus on the acute effects of orexin, ignoring the relevance of long-term effects. Therefore, future studies should incorporate chronic models and further investigate the role of the orexin system in different subtypes of depression to obtain findings with greater clinical relevance.

4. Relationship between orexin and depression in clinical research

Considering the progress of animal model studies in clarifying the regulatory role of the orexin system in depression, the next logical step is to translate these findings into clinical applications. Although clinical research into the relationship between orexin and depression is relatively recent, some studies have started investigating the correlation between orexin levels and depressive symptoms. The orexin system plays a crucial role in regulating arousal, wakefulness, and appetite and is implicated in the pathophysiology of depression. Reportedly, the orexin system dysregulation contributes to the development of depressive symptoms, including sleep disturbances predominantly associated with depression.[69] We review the existing clinical research to explore the role of the orexin system in human depression.

Orexin possesses the ability to control hormone levels, eating habits, and sleep–wake cycles and may be closely involved in the pathophysiology of major depressive disorder (MDD), a severe mood disorder potentially influenced by abnormalities of neurotransmitters in orexin neurons. Studies have determined whether the OX1R and OX2R genes contribute to the onset of MDD. A study comparing 87 healthy controls and 75 patients with MDD revealed that substantial differences were found in the genotype frequencies of the OX1R gene variants rs10914456 and rs2271933, whereas the Orx2 rs2653349 genotype showed no association with MDD development. The rs10914456 and rs2271933 loci of the OX1R gene may be related to the pathogenesis of depression. The OX1R rs10914456 variant may affect the severity of depressive symptoms.[70]

According to preclinical research, a strong correlation exists between orexin and stress-related diseases. In addition, orexin is involved in regulating animal reward and motivation,[71] inferring that a potential association exists between the orexin energy system and depression in human subjects. Numerous studies in adults have indicated a negative correlation between MDD and orexin levels.[72,73] Nevertheless, these studies have primarily focused on adults, with limited research conducted on adolescents. Considering the distinctions between adult and adolescent depression,[74] as well as the understanding that orexin receptor expression tends to decrease with age,[75] the association between MDD and orexin levels could differ among adolescents. Serum OXA levels were compared between adolescents with MDD and healthy controls using ANCOVA; in the MDD group, correlation analysis and linear regression were also used to analyze the correlation between OXA levels and the childhood depression scale. After controlling for body mass index, sex, age, and anxiety levels, OXA levels in depressed individuals were comparable to those of controls. Moreover, neither correlation nor regression analysis revealed an association between OXA and depressive symptoms, implying that OXA was not related to adolescent depression.[76]

Childhood abuse results in neuroendocrine alterations, which may be associated with an increased vulnerability to psychiatric pathology, such as depression and anxiety.[77,78] Reportedly, the relationship between childhood abuse and orexin levels in patients with depression and anxiety was investigated using the Childhood Trauma Questionnaire (CTQ) to evaluate the history of childhood trauma in 27 women with depression or anxiety symptoms and 27 healthy women controls. Serum levels of orexin and cortisol were measured in all participants.[79] Results showed that orexin levels in patients' serum positively correlated

with the CTQ total score and some CTQ subscale scores, such as emotional neglect. In the control group, the scores for emotional neglect positively correlated with serum orexin levels. Meanwhile, there were no notable differences in serum orexin and cortisol levels between patients and controls, suggesting that orexin levels are associated with childhood abuse and not with psychopathology such as depression or anxiety.

Although the orexin system regulates mood and stress responses, its role in adolescent depression remains underexplored. Comprehensive data on age-specific orexin expression patterns present a remarkable gap in the literature, particularly for the developmental changes in the orexin system. Additional experiments into targeted therapeutic strategies for age-related depressive disorders are required to fill this gap.

Furthermore, the impact of sex differences on orexin signaling and depression has been documented, with studies indicating that inherent differences in the orexin system may contribute to the higher prevalence of depression in women.[80] Women with depression exhibit significantly higher levels of orexin-immunoreactivity (ir) than men with depression. Moreover, the daily fluctuation pattern identified in the hypothalamic orexin-ir was not detected in individuals with depression. Men with depression who died by suicide exhibited substantially increased OX2R-mRNA expression in the anterior cingulate cortex compared with men in control. This finding suggests the need to consider the definite sex-related alteration observed in the hypothalamus OXA-ir in depression in the development of orexin-targeted therapy methods.[81]

To summarize, the relationship between the orexin system and depression is an emerging research field. Current studies primarily focus on investigating their correlation, whereas there is a lack of intervention studies targeting the orexin system. Future research could utilize longitudinal study designs to track changes in orexin levels over time and evaluate their causal relationship with the onset and progression of depression. In addition, incorporating techniques such as neuroimaging and neurophysiology could provide insights into the relationship from different perspectives by tracking changes in the orexin system during the onset, progression, and treatment of depression and revealing its dynamic mechanisms of action.

5. Orexin- and depression-related neurological diseases

The orexin receptor pathway plays a role in the pathophysiology of various nervous system disorders, such as drug addiction, narcolepsy, depression, and Alzheimer's disease. Preclinical studies indicate that the orexin neuropeptide system influences wakefulness and various behaviors using a network of axons that project from the hypothalamus to multiple, sometimes distant, regions of the brain. Different forms of incoming information are integrated by orexin neurons and transformed into the necessary behavioral output in conjunction with appropriate arousal state.[82]

Narcolepsy is caused by the depletion of hypothalamic orexin-producing cells.[83] Individuals diagnosed with narcolepsy had undetectable levels of OXA in their cerebrospinal fluid and frequently experienced comorbid depression.[84-87] Patients with depression and narcolepsy have short rapid eye movement sleep latency. A previous study evaluated OXA levels in 15 individuals with depression and 14 controls. Under entrained light–dark circumstances, the cerebrospinal fluid was continuously extracted from supine participants for 24 h using an indwelling intrathecal catheter. Patients with depression were examined before and after receiving the antidepressants sertraline and bupropion for 5 weeks.[88] Results showed slight changes in OXA levels (10%) in the control group; however, the levels remarkably changed throughout the day and night cycle and notably decreased in patients with depression (3%). The levels were the lowest at noon, which was surprising for a hypothetical peptide that promotes sobriety. The average level of hypothalamic secretion was higher in patients with depression than in control subjects. After treatment with both drugs, the OXA level in the sertraline group showed a slight but notable decrease (14%), whereas the bupropion group showed no changes in OXA levels. Sertraline is a selective serotonin reuptake inhibitor that primarily acts on the serotonin system.[89,90] Because an interaction exists between the serotonin system and orexin neurons, sertraline may indirectly affect the release of OXA by regulating serotonin levels.[91] Bupropion primarily acts on the noradrenergic and dopaminergic systems, with minimal direct impact on the serotonin system.[92] Hence, bupropion and sertraline may affect OXA levels differently. These data are consistent with physiological findings on depression, indicating that the diurnal changes in OXA levels are inhibited.

The states of sleep and wakefulness emerge from the intricate interactions within the sleep–wake circuitry.[93] Drowsiness is caused by long-term signaling abnormalities in orexin neurons, which are most active when awake and silent when inactive.[94] Kleine–Levin syndrome (KLS) is a rare recurrent drowsiness disorder. In a clinical evaluation, 44 of 57 individuals with recurrent drowsiness fulfilled the behavioral and clinical requirements for KLS. In a subgroup of individuals, the diurnal blood pressure and

OXA levels in the cerebrospinal fluid were evaluated in relapse versus remission. The heart rate and blood pressure fluctuated during the symptomatic phase and were lower during remission, suggesting an involvement of orexin dysregulation in KLS pathogenesis.[95] To investigate the correlation between OXA and sleep in obese patients, orexin content and sleep were evaluated in 26 participants with obesity, 40 participants who were pathologically obese, and 32 normal weight participants. Structural equation modeling revealed that plasma OXA levels were associated with decreased overall sleep quality. The results support a link between high plasma OXA concentrations and insufficient sleep, which may exacerbate depression.[96]

An increasing body of research link orexin disturbances to a number of neuropsychiatric conditions, such as addiction, anxiety, and depression. Several psychiatric conditions share similarities with the behavioral variant frontotemporal dementia (bvFTD) syndrome. A study evaluated the OXA concentrations in the cerebrospinal fluid of 40 patients with bvFTD and 32 non-demented individuals and correlated them with various clinical features. There was a remarkable elevation in OXA concentrations among patients with bvFTD compared with those in the control. OXA concentrations in the cerebrospinal fluid correlated with the Mini-Mental State Examination scale score, medication hypothesis, history of compulsive behavior, and extrapyramidal signs. These data provide evidence of orexin dysfunction in patients with bvFTD associated with depression.[97]

Orexin exerts considerable effects on the neurophysiological and behavioral aspects of emotional illnesses. However, there is limited research on alterations in orexin levels among individuals with emotional problems. In one study, the plasma level of OXA was measured in individuals with mood disorders and a control group using the enzyme-linked immunosorbent assay. Patients with bipolar disorder (BD) and MDD showed considerably higher plasma levels of OXA. Moreover, plasma OXA levels of the BD group were remarkably higher than those of the MDD group. Individuals in the MDD group who thought about suicide more often had higher OXA levels than those who thought about suicide less frequently.[98] Thus, the distinct variations in plasma OXA levels might differentiate between MDD and BD and diagnose depression. The distinct alterations in OXA levels associated with suicidal ideation in depression may prevent suicidal behavior and is considerable in the ongoing research on orexin-targeted therapeutics.[98]

The pathophysiology of mental illnesses has been associated with changes in the orexin system. There have been attempts to determine whether plasma orexin concentrations in patients with BD, schizophrenia, or MDD differ from those in the healthy control group. Moreover, a potential relationship between plasma OXA levels and clinical characteristics was investigated in a study of 80 healthy controls, 80 patients with schizophrenia, 80 patients with MDD, and 40 patients with BD. Plasma OXA levels were measured using an enzyme-linked immunosorbent assay, showing that the mean OXA levels of the four diagnostic groups considerably varied. Specifically, patients with BD had far lower levels of OXA than controls. The study found no correlation between plasma OXA levels and pharmaceutical dosages, depression severity, or any clinical symptoms, implying that patients with BD had lower plasma OXA levels.[99,100]

According to research, the symptoms of Parkinson's disease frequently include sadness, hallucinations, sleeplessness at night, daytime sleep episodes, and rapid eye movement sleep behavior disorder. Narcolepsy presents various symptoms related to a specific depletion of orexin neurons. A previous study investigated the functionality of the orexin system in individuals with Parkinson's disease to determine any potential dysfunction in orexin cells. The hypothalamus of 11 patients with Parkinson's disease and 5 normal controls was examined, revealing that the loss of hypothalamic secretin cells increased with the progression of the disease. Similarly, the loss of MCH cells increased as the illness worsened. The entire anterior to posterior region of the hypothalamus distribution showed a loss of orexin and MCH cells. A significant loss of orexin neurons is a hallmark of Parkinson's disease. Therefore, therapies targeted at correcting orexin deficiencies can mitigate depression, which may be caused by the loss of orexin cells.[101]

To summarize, dysfunction of the orexin system is manifested in various neurological disorders, providing a scientific basis for the development of treatments targeting depression and related neurological diseases. Considering the overlap between the inflammatory processes of depression and neurodegenerative disorders, the current research lacks exploration into the bidirectional relationship between orexin signaling and neuroinflammation.

6. Regulatory process of depression mediated by orexin

The orexin/receptor system is essential for regulating various physiological functions such as sleep–wake cycles, reward processing, feeding behavior, addiction, and energy balance. The regulation of energy expenditure is related to the active hypothalamic nerve mechanism that controls adaptive stimulation.[102] Extensive research has demonstrated the participation of orexin/receptor

pathways in the pathogenesis of neurological conditions.[103] Reportedly, the orexin/receptor system can be investigated as a promising treatment focus for substance use disorders.[104] Moreover, the pathophysiology of MDD involves increased orexin signaling, although the precise relationship between the orexinergic system and depressive symptoms remains unclear. OXA is related to the HPA axis, which regulates the stress system and response.[105] A study investigated the potential antidepressant-like effects of treating mice exposed to unanticipated chronic moderate stress by blocking orexin receptors, as well as the underlying mechanisms. The findings indicate that the drug blocking the orexin system can exert strong antidepressant effects and induce the recovery of stress-related HPA axis defects independent of neurogenic effects.[50]

As mentioned earlier, the orexin system includes two neuropeptides (OXA and OXB) and two G-protein-coupled receptors (OX1R and OX2R).[106] Orexin receptor antagonists are a novel class of psychotropic drugs for treating insomnia and other psychiatric disorders such as depression.[107] Moreover, orexin receptor antagonists have therapeutic potential for mood disorders by modulating the expression of neuropeptides in the hypothalamus and limbic system.[108] The potential of orexin receptor antagonists, including dual OX1/2R antagonists (DORAs), selective OX1R antagonists (SORA1s), and selective OX2R antagonists (SORA2s), in treating depression will be reviewed subsequently. An analysis of preclinical and clinical data demonstrated that although SORA1s have the potential to treat drug addiction and anxiety,[109] SORA2s and DORAs exhibit great efficacy in treating sleep disorders and even depression.[108]

Existing evidence shows that for emotional behavior, OX1R and OX2R may have opposite functions. OX1R primarily promotes anxiety and depression, and the major potential of drug treatment related to OX1R is to block anxiety and depressive behaviors through antagonists. The effect of the intraperitoneal injection of the OX1R antagonist SB334867 on depression in mice was explored using the number of crossings of FST, tail suspension experiment, and wilderness experiment,[110] revealing that SB334867 exerted an antidepressant-like effect because it reduced the immobility duration in the FST without affecting the locomotor behavior.

Orexin neuropeptides stabilize arousal, and several orexin receptor antagonists have been approved for treating insomnia in adults.[111] OX2R is gaining recognition as a novel therapeutic target for addressing persistent insomnia in individuals with depression.[112] OX2R is deeply involved in controlling alertness, arousal, and sleep–wake cycles. Insomnia is often the result of physiological over awakening. Stimulating OX2R improves resilience to social stress, anxiety, and depression, whereas inhibiting OX2R promotes susceptibility to these conditions.[113] OX2R agonists have been demonstrated to promote psychological elasticity and antianxiety and antidepressant behaviors. Insomnia is often related to depression, and considering insomnia is a prevalent symptom of depression, OX2R antagonists may provide valuable therapeutic options for individuals with MDD.[114] Seltorexant is a selective OX2R antagonist, developed for treating MDD, and possesses sleep-promoting properties.[115,116] The pathophysiology of MDD includes overawakening. By selectively blocking the human OX2R, seltorexant can reduce the symptoms of depression by normalizing excessive arousal.[115] Seltorexant is a potential option for treating depression and anxiety considering that its use is safe and that it exerts no obvious or substantial adverse effects from a therapeutic viewpoint.[69,117] To assess whether a potent selective OX2R antagonist – TCS OX2 29 (TCS) – exerts a positive effect in an animal model of detrusor overactivity coexisting with depressive-like states in male rats, a related study conducted FST to measure the spontaneous locomotor activity, conscious cystometry, and c-Fos expression in central micturition areas of rats and performed several biochemical analyses.[118] Therefore, TCS (3 mg/kg/day, subcutaneous injection) administered for 7 days normalized the cystometric parameters corresponding to the overactivity of the detrusor and reversed the predepressive responses; this finding opens up a novel perspective on the role of the orexin system in bladder function and the pathophysiology of depression.

DORAs treat insomnia[119,120] by inducing drowsiness, which is achieved by blocking the wake-up-promoting effect of orexin neuropeptides. Several randomized clinical trials have demonstrated the effectiveness of DORAs in effectively treating chronic insomnia.[121,122] Suvorexant – which acts as an antagonist of orexin receptors – is used in clinical practice for treating insomnia. This treatment is based on the association between hyperactivity of the orexin system and sleep disorders.[123] A novel dual orexin receptor antagonist – daridorexant (ACT-541468) – is being investigated for treating sleeplessness, a common co-occurring condition with anxiety and depression.[124-127] In addition to treating insomnia, it treats cardiovascular problems, chronic obstructive pulmonary disease, and Alzheimer's disease.[128] Existing evidence provides valuable insights into the real-world safety profile of daridorexant, supporting the presence of safety concerns related to nightmares, depression, and hangovers.[129] Almorexant is a DORA that exerts sleep-enabling effects in humans.[130] Almorexant can inhibit the effects of OXA, which improves with its increasing concentrations.[131] In a previous study,

the combination of equilibrium and kinetics with the selective OX2R antagonist radioligand [^{3}H]-EMPA investigated the effects of almorexant. Based on an analysis using a hemiequilibrium model, the dissociation of antagonists in a biological system occurs at a slower rate than membrane binding. In such scenarios, almorexant acts as a pseudoirreversible antagonist.[132]

Another study using an animal model of depression showed that the pharmacological blockage of orexin receptors exerts a strong antidepressant-like effect.[133] The effect of intracerebral venous administration of OXA on FST and cell proliferation with bromodeoxyuridine (BrdU) in the dentate gyrus was investigated in another study. BrdU was used to label and quantify newborn neurons in the hippocampus. OXA administration notably reduced the immobility of animals in the FST without affecting spontaneous motor activity or serum cortical ketone levels. Moreover, the number of BrdU-positive cells in the dentate gyrus substantially increased in OXA-treated mice. Treatment with the OX1R antagonist SB334867 blocked the OXA-induced decrease in immobility in the FST and the increase in BrdU positivity;[134] these findings show that OXA exerted an antidepressant effect.

Although preclinical studies on orexin receptor antagonists indicate antidepressant effects, a remarkable limitation is the reliance on short-term animal models, which may not completely capture the chronic nature of depression. Longitudinal studies incorporating long-term or chronic intervention models are required to gain clinically relevant insights.

Pharmacological evidence suggests a link between the orexin, monoamine, and cannabinoid systems in appetite and emotional behavior control.[135] The orexin and endocannabinoid systems exhibit numerous shared biological functions, encompassing arousal, stress response, mood regulation, and reward processing.[136] Reportedly, OXA may activate postsynaptic OX1R and induce 2-AG production,[137] a mechanism achieved through the Gq-protein-mediated PLC-DAGL enzyme cascade.[138] The activation of G-proteins activates downstream signaling pathways, suggesting that the orexin/receptor system can activate effective intracellular signaling through these signaling pathways (Figure 3).

Chaihu-Jia-Longgu-Muli decoction (CLMD) is an ancient prescription documented in the Treatise on Febrile Diseases by Zhang Zhongjing, the renowned figure in traditional Chinese medicine. CLMD has been widely used and has demonstrated its efficacy in ameliorating chronic inflammatory condition in mice with chronic kidney disease. Evidently, CLMD intervention may regulate energy metabolism and improve sleep quality and cognitive function in mice with chronic kidney disease. The mechanism underlying this effect may involve the upregulation of OXA expression and the augmentation of CaMKKβ/AMPK phosphorylation. These changes subsequently inhibit the downstream signaling pathway of nuclear factor-κB, reduce the production of inflammatory cytokines such as interleukin-1β (Figure 3), and reduce inflammation, thereby ameliorating the inflammatory state associated with central and peripheral system disorders[139] and further affecting the onset, development, and treatment of depression. Exogenous OXA administration substantially improved lung histology and reduced inflammation levels, emphasizing the importance of neuroimmune interaction and suggesting that OXA holds promise as a therapeutic agent for acute lung injury.[140]

The development and occurrence of psychosis are closely associated with the functional modulation of the orexin system in the CNS. Depression and physical symptoms are linked to abnormal alterations in the hypothalamic lateral region. The pathophysiology of depression may be revealed through traditional Chinese medication Xiaoyaosan that controls these alterations. A depressed rat model was established to observe the changes in the OXA/OX1R expression in the LH and the intervention of Xiaoyaosan; Xiaoyaosan could considerably reverse the expression of OXA/OX1R in the LH, and the curative effect was remarkable. The pathophysiology of depression and somatic symptoms is intimately pertaining to the aberrant alterations of OXA/OX1R in the LH of depressed rats induced by prolonged stress. By controlling OXA/OX1R, Xiaoyaosan could alleviate physical complaints and depression.[141]

Light intensely affects the behavior and physiology of almost all animals, including humans. Reportedly, exposure to relatively dim light throughout the day was associated with increased depression- and anxiety-like behaviors and impaired spatial memory. In contrast, bright light during the day reduced depressive and anxious behaviors along with improving spatial memory.[142] Orexin is a vital mediator of these effects because it responds to changes in light levels during the day and transmits this information to other brain regions associated with emotional behavior and spatial memory (Figure 7).

Maternal and infant separation (MIS) is a mature depression model. Reportedly, chronic continuous light (CCL) treatment during puberty can effectively mitigate depression-like behavior triggered by MIS. Long-term light altered the serotonin and orexin system in the brain of rats. The speculation from such studies is that a certain relationship exists between serotonin and orexin system in depression. Low concentrations of orexin exert a direct

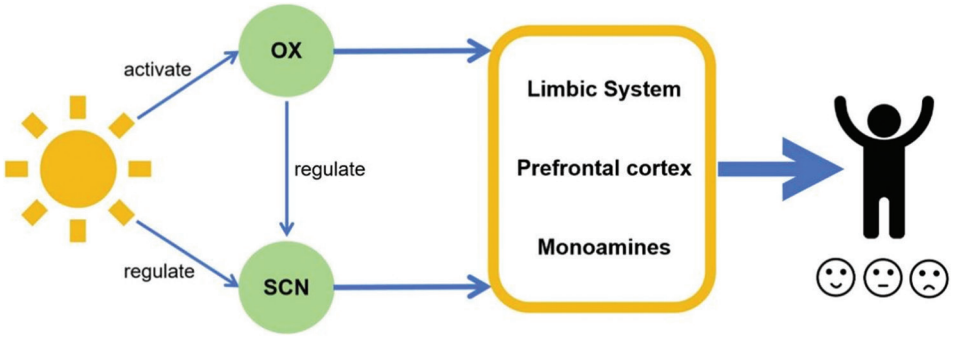

Figure 7. Relationship between sunlight, orexin, and emotion. Light regulates the suprachiasmatic nucleus through the retina-hypothalamic signaling pathway and indirectly affects the orexin system by modulating the neurotransmitters and hormones interacting with orexin. Orexin neurons project to multiple brain regions, regulating mood and cognition by influencing the suprachiasmatic nucleus activity in the circadian rhythm. Altogether, these mechanisms modulate the monoaminergic system, limbic system, and prefrontal cortex, which are crucial for regulating mood and cognitive processes. Abbreviations: OX: Orexin system; SCN: Suprachiasmatic nucleus.

excitatory effect on serotonin neurons, whereas high concentrations indirectly inhibit them[103,143] (Figure 4) because low orexin concentrations act on its receptors OX1R and OX2R in serotonin neurons, possibly exciting them by regulating the activity of these receptors;[91] however, at higher concentrations, orexin may indirectly inhibit serotonin neurons by exciting GABAergic interneurons.[144,145] In another study, adult male rats were used to induce MIS-related alterations. A subset of these rats were treated with CCL for 3 weeks during puberty. Results demonstrated that CCL therapy affects the serotonin and orexin energy systems in the MIS model but does not affect the reduction of dopamine release in the MIS-induced nucleus accumbens; this observation suggests that bright light is associated with improved mood and cognitive function. Therefore, restoration of the orexin system could be associated with the antidepressant effects of CCL treatment in cases of depression.[146]

Early life stress contributes to the onset of depression. While dealing with stress, orexin neurons drive awakening and motivational behaviour.[147] Although physical exercise is widely recognized as a beneficial intervention, the precise mechanism of action remains incompletely understood. To explore the antidepressant effects of exercise, researchers have investigated the involvement of the amygdala, specifically the BLA, in animal models of depression. Repeatedly bound mice exhibited depressive behavior, which could be offset by post-stress treatment (exercise). After repeated stress, the hypothalamic secretion hormone/orexin hormone and MCH were upregulated in the BLA, whereas exercise therapy could reduce stress-induced depression-like behaviors; these findings indicate that exercise exerts an antidepressant effect by suppressing the orexin and MCH neural pathways within the BLA.[148] The production of microcirculation and growth factors are most likely involved in movement-induced changes in the hippocampus. The orexin system may be related to locomotor behaviour,[149] and a speculative factor that promotes the beneficial effect of exercise is OXA. Similarly, previous research has documented that anorexigenic neurons are closely linked to brain areas implicated in cognitive function and emotional regulation, OXA level increases with exercise,[150] and OXA may affect the synaptic plasticity of hippocampal neurons and hippocampal neurogenesis by regulating lactate metabolism, thereby affecting cognitive function.[49] Promoting hippocampal neurogenesis is a potential strategy for improving cognitive function and treating neuropsychiatric disorders such as depression. Hence, the beneficial effect of exercise may involve preventing the decline of cognitive function caused by degenerating the hippocampus associated with aging, to improve depressive symptoms.

7. Conclusion

Depression is a common mental health condition that impacts an individual's well-being and overall health. Recent research has highlighted the critical involvement of orexin in the onset and development of depression. Patients with depression exhibit lower levels of orexin than those without depression, along with alterations in the expression of orexin receptors. The orexin/receptor system is involved in various functions within the CNS, which is enabled by the extensive projections of orexin-containing neurons, complex circuitry involving other neuron types, and widespread distribution of orexin receptors. Orexin inhibits the release of GABA (Figure 2), which may increase the excitability of orexin neurons that depends on the distinct functions of $GABA_A$ and $GABA_B$ receptors, the distribution of these receptors at presynaptic and postsynaptic locations, and their

expression in specific neural circuits. $GABA_A$ receptors are located in the presynaptic terminals,[151] and their activation allows Cl^- to flow into postsynaptic neurons and induce inhibitory postsynaptic potentials.[152] In contrast, $GABA_B$ receptors are distributed at presynaptic and postsynaptic sites, and their functions typically involve the regulation of neurotransmitter release and neuronal excitability, with their role at the presynaptic site closely associated with spinal presynaptic inhibition caused by primary-afferent depolarization. Consequently, decreasing GABA release may disturb the excitatory–inhibitory balance, which is particularly evident in the interaction between glutamatergic and GABAergic neurons.[153,154] Orexin increases dopamine and regulates reward and motivational behavior through interactions with dopamine receptors. In addition to its involvement in regulating mood and the sleep–wake cycle, orexin is implicated in various aspects of stress response and substance abuse. For instance, orexin interacts with the noradrenaline system and HPA axis, regulating the HPA axis by increasing the release of norepinephrine (Figure 5) and modulating stress responses and improving alertness. The hypothalamic-ventral tegmental pathway is a crucial regulator of appetite and energy balance. Within this pathway, the activity of orexin neurons substantially promotes feeding behavior. Animals that lack orexin experience a loss of appetite and subsequent weight loss. The hypothalamic-ventral thalamic pathway also considerably regulates reward behavior and drug abuse. The activity of orexin neurons can improve reward behaviors and participate in drug craving and abuse behaviors.

Nonetheless, the mechanism of action of orexin in depression remains unclear, and a lack of such data is the biggest obstacle to a comprehensive understanding of the pathophysiology of depression. Further, exploring the interactions between orexin and other neurotransmitters and signaling pathways, as well as its specific role in the development of depression, is necessary. Moreover, depressive symptoms can be improved by regulating the orexin system, and orexin receptor antagonists have demonstrated potential to treat depression. Therefore, developing more selective and efficient orexin receptor antagonists is a crucial focus for future research.

Overall, although orexin has demonstrated promise in depression studies, understanding its role across depression subtypes remains challenging. Future research should focus on clarifying the distinct effects of orexin in atypical and melancholic depression, as well as its association with comorbid conditions such as anxiety, which may present different responses to orexin-targeted treatments. This article provides some suggestions for the research, treatment, and prevention of mental diseases such as depression through a comprehensive review of orexin and depression.

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Conflict of interest

The authors declare no conflicts of interest.

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No.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Sex differences in autoimmune disorders: Inspecting the roles of the X chromosome

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Abstract

Autoimmune disorders are complex, heterogeneous conditions that can severely impact an individual's quality of life. These diseases are associated with a breakdown of central and peripheral processes controlling self-tolerance, causing the presence of circulating autoreactive immune cells that target the body's own cells and tissues. Some data suggest that autoimmune diseases (ADs) are becoming increasingly prevalent in modern society. Possibly, both genetic and environmental factors contribute to the rise. ADs disproportionally affect females compared to males. Hormonal determinants, particularly sex-steroid hormones, have historically been proposed as key modulators of the differential susceptibility to ADs between the mammalian sexes. Emerging evidence has more recently generated significant focus on the X chromosome as a potential key player in ADs pathogenesis. The X chromosome, one of the largest chromosomes in the mammalian genome, exhibits a different pattern of inheritance between the sexes. In females, one X chromosome is typically silenced in somatic cells to balance the active X dosage between the sexes. The X-inactivation process is not fully efficient as a proportion of X-linked genes is capable to escape silencing and maintaining variable, biallelic expression degree within each cell. Notably, the X chromosome is rich in genes related to immune functions; variations in X chromosome dosage can alter the susceptibility of developing autoimmune traits. Both X-linked genes and X-linked mechanisms have been associated with ADs. In this review, we discuss the X chromosome's crucial roles in ADs.

Keywords: Autoimmune diseases; Sex differences; X-chromosome inactivation; Skewed X-inactivation; Escape from X-inactivation; Systemic lupus erythematosus; Sjogren's syndrome; Hashimoto's thyroiditis

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1. Introduction

Autoimmune diseases (ADs) are a diverse class of conditions arising from aberrant immune responses against individual's own molecular and cellular constituents. ADs

are characterized by altered lymphocytic T and B cells reactivity.[1] ADs are complex traits, exhibiting high variability across individuals. ADs arise from a breakdown in central and peripheral self-tolerance controls leading to the survival and proliferation of autoreactive immune cells that cause autoimmunity. ADs include tissue/organ-specific and systemic inflammatory disorders.[2] It is estimated that about 10% of the worldwide population is affected by ADs, with a striking predominance among females. ADs may be on the rise,[3-5] therefore representing a major global health and financial concern.

Typically, females exhibit stronger immunological response – such as to infections – compared to males. For instance, production of antibodies and immunoglobulin M is enhanced in females.[6-8] However, this enhanced immune response, which presumably results from a complex interplay of genetic and hormonal factors, comes at a cost: females are also at higher risk for developing ADs. There is a generalized higher incidence of ADs among females compared to males. Systemic lupus erythematous (SLE) presents a female-to-male ratio of 9:1.[9] For Hashimoto's thyroiditis (HT), the female-to-male ratio can reach 10:1.[10] Hormonal factors, such as estrogen and testosterone, have long been recognized as determinants of ADs risk, being implicated in immune responses and often exhibiting alterations in immune-related conditions, especially ADs.[11-13]

In recent years, the X chromosome has garnered significant focus in the study of ADs. Females may benefit from the presence of two X chromosomes as heterozygous genetic variation may enhance phenotypic diversity, such as increased variability in immune-related responses. The X chromosome is abundant in genes related to immune functions.[14,15] The genetic and consequent functional imbalance in X-linked gene dosage between the mammalian sexes leads to the hypothesis that the X chromosome may play critical roles in risk and pathogenesis of ADs, and gender differences in Ads.[16] Because females typically have two X chromosomes while males are typically hemizygous for X-linked genes, it was hypothesized that the X chromosome dosage may correlate with a higher risk to ADs. Supporting this hypothesis, males with 47, XXY karyotype (Klinefelter's syndrome) carry significantly higher risk – up to 14-fold higher for SLE – of ADs compared to typical 46, XY males.[17] Females affected with triple-X syndrome (47, XXX) may also exhibit heightened predisposition to certain ADs compared to 46, XX females[18] (Figure 1). This suggests that while having two X chromosomes may confer females with a more robust immune system than males, it simultaneously increases their susceptibility to ADs.

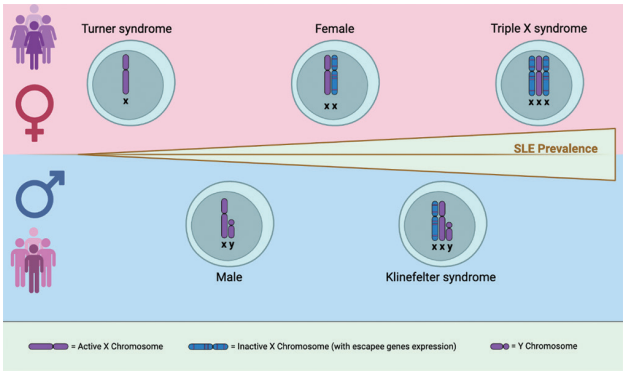

Figure 1. The impact of X chromosome dosage on systemic lupus erythematosus prevalence. Epidemiological and clinical data suggest that systemic lupus erythematosus (SLE) risk correlates with X-dosage in both the female and the male sexes. Distinct sex chromosome karyotypes are shown, including Turner syndrome (45, X), typical females (46, XX), triple X syndrome (47, XXX), typical males (46, XY), and Klinefelter syndrome (47, XXY). The central gradient represents the increasing susceptibility to SLE correlated with the rising X chromosome dosage. Each circle indicates a cell. Created using BioRender.

Extensive research has explored the molecular mechanisms by which X-linked genes may influence the functions of the immune system.[16,19,20] In this review, we delve into the roles of the X chromosome in ADs, summarize findings regarding the involvement of X-linked genes in major autoimmune conditions, and discuss potential avenues for future research.

2. The complex biology of the X chromosome

The mammalian X chromosome is a genetic and epigenetic model.[21] It is one of the largest chromosomes in both the human and mouse genome. The human X chromosome spans about 155 Mb and carries over 800 protein-coding genes.[14] The X-linked dosage differs between XX female and XY male mammals. Typically, XY males inherit the maternal X, while XX females also inherit the paternal X chromosome. The X chromosome inactivation (XCI) process has evolved in mammals to balance the disparity in X-dosage between the sexes.[22,23] XCI epigenetically silences one X chromosome in each female somatic cell to balance the active X dosage between the sexes. Once established, the inactive X chromosome within a cell is stably passed on to daughter cells. XCI is a multi-step process involving a complex interplay between non-coding RNAs and proteins. It is highly regulated in time and space, with multiple aspects yet to be characterized.[24]

The choice of the X chromosome designated for inactivation is stochastic. This would result in somatic mosaicism of cells with either parental X silenced.

However, skewed (i.e., asymmetric) representation of a parental, active X chromosome is commonly seen among females, particularly in the aging population.[25-27] Both genetic and non-genetic factors can contribute to skewed XCI.[25,28] At present, the exact identity of these factors is not known. Skewed XCI impacts the somatic XCI mosaicism across cells and tissues. When restricted to certain immune cell populations, skewed XCI could potentially modulate differences in X-linked gene expression between cells, and consequently, impact the functions of the immune system. Skewed XCI also occurs in the presence of X-linked mutations, as in the case of asymptomatic female carriers of Wiskott-Aldrich syndrome where the wild-type allele is preferentially expressed in hematopoietic cells.[29] Crucially, the inactive X chromosome is not fully silent. In humans, more than 20% of X-linked genes "escape" XCI and exhibit partial-to-complete expression from both parental X chromosomes in a female somatic cell.[30-34] For genes regulating immune system functions, escape from XCI might increase their functional dosage and underlie sex differences in immune phenotypes.[33,35,36] As discussed below, X-linked genes escaping XCI have been associated with ADs.

3. Sex-biased ADs risk: A female-specific disadvantage

Epidemiological and clinical data indicate that the female sex is at significantly higher risk for ADs compared to the male sex (Table 1). In general, up to 80% of individuals with a clinical diagnosis of an ADs are women. In discussing the involvement of X-linked genes in ADs, we will focus on three prominent, sex-biased ADs: Systemic lupus erythematosus, Sjögren's syndrome, and HT, all of which show a prevalence reaching 80% in female patients.

3.1. SLE

SLE is an autoimmune, systemic disease. Up to 90% of diagnosed patients are female.[9] The female sex and karyotypes with extra X chromosomes are at higher risk of SLE. SLE is a highly heterogenous disease. Clinical manifestations may range from mild mucocutaneous expressions to inflammatory involvements of the central nervous system as well as multiple other organs. The exact etiology of SLE remains not well understood. At molecular level, anti-nuclear autoantibodies and Type I interferon (IFN) activity are detected. Multiple anomalies of B- and T-cell activity have been characterized in SLE.[37-40] T-cells exhibit aberrant maintenance of XCI in individuals affected with SLE as well as in NZB/W F1 XX mice, which develop a systemic, ADs highly similar to SLE.[41] Up to a hundred X-linked genes are overexpressed in T-cells from SLE patient,[41,42] presumably resulting from X-reactivation.

Table 1. Female prevalence for a subset of systemic and organ-specific autoimmune diseases

	Female: Male ratio	Category	References
Systemic conditions			
Sjögren's syndrome	20:1	Systemic	63
Systemic lupus erythematosus	9:1	Systemic	9
Takayasu's arteritis	9:1	Vasculitis	144
Systemic sclerosis	3:1	Systemic	145
Giant cell arteritis	3:1	Vasculitis	144
Rheumatoid arthritis	3:1	Systemic	146,147
Organ-targeted conditions			
Hashimoto's thyroiditis	4 – 10:1	Thyroid	10
Grave's disease	4:1	Thyroid	148
Multiple sclerosis	3:1	Neurological	149

Among the upregulated genes, there are genes normally subject to XCI, such as *FOXP3* and *IL2RG*, but also known escapees such as *KDM5C* and *JPX*.[41] As discussed later in this review, multiple other X-linked loci have been significantly linked to SLE pathogenesis and susceptibility, such as Toll-like receptor 7 (*TLR7*), *CXorf21*, *TMEM187*, and *IRAK1*, which all escape XCI. By escaping XCI, these genes may exhibit partial to complete biallelic X-linked expression in female's immune cells, contributing to gender differences in risk of ADs.[32,33,35]

3.2. Sjogren's syndrome (SS)

SS is a systemic, chronic ADs which primarily affects the lacrimal and salivary exocrine glands. Compared to other ADs, SS is more frequently associated with multiple types of lymphomas.[43,44] As for other ADs, age is a risk factor, with most SS patients being at least 40 years old. There is no approved therapy to cure SS. The determinants of SS remain under active investigation. While the presence of a concurrent ADs, such as SLE or rheumatoid arthritis, appears to boost the risk of developing SS, genetic predisposition to SS is supported by cases of families with multiple affected members.[45-51] Genome-wide association analyses for primary SS uncovered consistent signal within *HLA* genes, particularly at MHC Class II locus *HLA-DRB1* in the Caucasian population.[52,53] Larger studies, also including multiple ethnicities, provided more detailed genetic associations at *HLA* Class II alleles DRB1 × 03:01, but also *HLA-DQA1* and *HLA-DQB1*.[54-57] Beyond MHC genes, other genes associated or potentially associated with SS have been reported, including IFN regulatory factor 5 (*IRF5*),[58,59] a transcription factor activating *IFNA/B* regulated also by *TLR7-9*; interleukin 12A (*IL12A*),[60] a cytokine required for T-cell induction

of IFN gamma (IFN-γ) and T-cells differentiation; signal transducer and activator of transcription 4,[58,61] which is activated by IFN and regulates lymphocytic response to *IL12* and T-cell differentiation; and oligoadenylate synthetase 1,[62] another IFN-regulated genes, known for its involvement in apoptosis and frequent overexpression in autoimmune phenotype. Notably, similar to current knowledge about biochemical signaling in SLE, most of these genes are involved in molecular processes of response to IFN. It is known that the primary biological risk factor for SS is the female sex. SS is the most female-biased autoimmune disorder, with a female-to-male sex ratio up to 20:1.[63] Females carrying a 47, XXX karyotype seem to harbor an increased risk of SS compared to typical, 46, XX females.[18] Males affected carrying a complete or partial 47, XXY karyotype (Klinefelter's syndrome) harbor a similar risk of developing SS compared to typical 46, XX females.[64] Altogether, these epidemiological data support a prominent role for the X chromosome dosage on the risk of SS. Interestingly, rare structural X-linked aberrations involving a partial segment triplication within the Xp11 region were identified in genetic analyses of over 2,000 women with SS.[65] Although a larger patient sample size is needed to identify additional cases, these genetic anomalies may pinpoint risk regions – including protein-coding and non-coding RNAs – that are functionally sensitive to X-dosage and involved in SS pathogenesis and pathophysiology.

3.3. HT

Similar to SLE and SS, HT (also known as chronic lymphocytic thyroiditis) exhibits among the most significant female-biased incidence. HT is characterized by auto-antibodies against the main thyroid antigens: Thyroid peroxidase and thyroglobulin.[66] These autoantibodies attack the thyroid gland, with lymphocytes infiltrating the thyroid and causing inflammation and gradual destruction of thyroid tissue. HT often causes hypothyroidism, where the thyroid fails to produce sufficient levels of hormones.[67] The exact nature of the molecular determinants of HT etiology remains not completely understood. The disease predominantly affects women, with a higher incidence in those aged 30 – 50. There is evidence on the genetic susceptibility of HT. Twin studies calculated that over 70% of the risk of developing thyroid autoantibodies can be attributed to genetics.[68] Earlier analyses of the sibling risk ratio confirmed that the development of HT has significant genetic components.[69] Later analyses of families revealed that children and sibling of individuals affected with autoimmune thyroid disease carried a 32-fold and 21-fold higher risk of developing HT, respectively.[70] Association studies and functional genomics screenings revealed multiple HT susceptibility genes, including *CTLA-4* (chr2q22), *PTPN22* (chr1p13), and *VDR* (chr12q13).[67] Several lines of evidence highlight the possible roles of the X chromosome in HT. First, HT is female-biased in prevalence. Second, X-monosomy (45, X0) in peripheral white blood cells – predominantly T and B lymphocytes – was found to be more frequent in females affected with autoimmune thyroid disease compared with healthy females.[71] Third, skewed XCI was found more common in HT subjects than healthy controls.[72,73]

4. X-linked genes and mechanisms involved in the pathogenesis of sex-biased ADs

The mammalian X chromosome plays a crucial roles in immune system functioning and is believed to significantly influence sexual dimorphisms observed not only in the risk but also in the expressivity of ADs.[16] Several X-linked primary immunodeficiencies have been characterized. Representative examples are Wiskott-Aldrich syndrome, caused by mutations in *WAS*[74]; X-linked agammaglobulinemia, caused by mutations in *BTK*[75]; and X-linked severe combined immunodeficiency.[76] These conditions predominantly affect males. The impact of increased X chromosome dosage on the risk of ADs is notable, as observed in X-linked aneuploidies. 47, XXY males exhibit substantially higher AD risk compared to 46, XY males.[17] Similarly, 47, XXX females may also face an increased risk of ADs compared to 46, XX females.[18] Data regarding Turner's syndrome (45, X0) are mixed; some reports suggest that 45, X0 females are at lower risk of ADs,[18,77] while others reported autoimmune manifestations in 45, X0 females and higher risk of autoimmune thyroiditis and inflammatory bowel disease.[78-80] As the copy number of X-linked genes could impact disease risk, immune-related genes escaping X-inactivation, which show partial to complete biallelic X-linked expression, are potential contributors to disease risk and expressivity, as well as phenotypic variability among females carries of X-linked heterozygous conditions.[32,36]

Several distinct X-linked mechanisms have been proposed as determinant of ADs.[16] Skewed XCI is one such mechanism that may disrupt tolerance mechanisms.[16,81,82] According to this hypothesis, it is crucial for antigen-presenting cells to harbor balanced representation of both X-linked alleles. If subset of dendritic cells exhibit pronounced skewing in XCI, their ability to identify and eliminate autoreactive T-cells may be compromised, as biased toward maternal or paternal self-antigens. As a result, non-tolerized, autoreactive T-cells infiltrate and propagate into the hematopoietic system, triggering autoimmune reactions.[81] Higher XCI-skew has been observed in individuals affected with rheumatoid

arthritis and autoimmune thyroid disease compared to healthy controls.[25,83] Patients affected with scleroderma also exhibited skewed XCI in blood cells.[84] Interestingly, divergent patterns across different diseases highlight the biological complexity of this phenomenon as well as possible inter-individual variability across autoimmune conditions.[85,86] As a representative example, a recent work found reduced XCI-skew in females affected with SLE compared to controls.[87] These variations may reflect distinct molecular mechanisms at play within different ADs, including heterogenous, cell-type specific impact on XCI-skew. ADs are characterized by excessive systemic or localized inflammation, often accompanied by proliferation of specific cell types. Autoreactive T-cells that evade immune system's tolerance in the thymus during early development may proliferate and differentiate both before and during autoimmune responses. For instance, in both murine models and human cases of SLE, hematopoietic stem and progenitor cells exhibit substantial expansion and enhanced differentiation potential on inflammation.[88,89] Distinct T-cell subsets, including regulatory T-cells, can also clonally expand upon stimulation in autoimmune responses.[90] Similarly, synovial fibroblasts, macrophages, and Th cells proliferate and differentiate in the synovium in patients with rheumatoid arthritis.[91] Therefore, due to enhanced cell proliferation, it is entirely possible that skewed XCI patterns in hematopoietic and immune cells could arise, to some extent, as a consequence of ADs.

Another proposed mechanism involves increased functional dosage of X-linked genes following X-linked reactivation. Epigenetic alterations of the inactive X chromosome may lead to the expression of genes that are typically silent, thereby contributing to autoimmunity. This hypothesis is bolstered by recent findings demonstrating that perturbations of XCI can reactivate X-linked genes, such as *TLR7*, in B cells, dendritic cells, and macrophages, with subsequent manifestation of lupus-like disease in mice.[92] Earlier studies have also indicated that reactivation of the silent X chromosome may contribute to SLE through the expression of CD40L in T cells, suggesting that specific epigenetic changes could act as pathogenetic drivers.[93]

The X chromosome harbors numerous immune-related factors, such as *TLR7/8*, *CD40L*, *IRAK*, and *FOXP3* – key players in innate and adaptive immune responses – and *BTK* and *IL2RG*, implicated in the development of immune cells.[94] Below, we will discuss a set of X-linked genes that have been associated with ADs.

TLR7 encodes for a member of the TLR family, which plays crucial roles in pathogen recognition and activation of innate immunity. *TLR7* is capable of recognizing ssRNA oligonucleotide sequences and triggering innate immune responses. On ligation with the target, TLR7 can signal the production of proinflammatory cytokines.[95] Through IRF7, TLR7 can also trigger production of Type I IFN by dendritic cells. Several studies have characterized the role of *TLR7* in the pathogenesis of SLE. *TLR7* genetic mutations, including gain-of-function mutations, have been reported are a cause of SLE.[96-98] Recent works identified a mutation in UNC93B1 – a TLR7 trafficking factor that interacts with ARL8B to regulate intracellular levels of TLR7 – as a possible cause of childhood SLE. Specifically, the mutation results in aberrant interaction with the BORC complex, which in turn leads to elevated endosomal TLR7 levels, triggering excessive immune stimulation.[99] Single-cell resolution analyses revealed that in XX cells and 47, XXY cells, *TLR7* escapes XCI in B lymphocytes, monocytes, and plasmacytoid dendritic cells. Cells with biallelic *TLR7* dosages also exhibited significantly greater potential of immunoglobulin G class switching.[100] These data support a role of *TLR7* escape, as well as other X-linked processes capable of modulating *TLR7* dosages, including XCI-skew, in ADs pathogenesis. A recent study found increased TLR7 expression in salivary glands of SS patients compared to healthy individuals. TLR8 KO mice develop SLE but also a SS-like pathology with ectopic lymphoid aggregates. Interestingly, when abrogating also TLR7 in TLR8 KO mice, the SS-like phenotypes are reduced, thus indicating an important role for TLR7 in the pathogenesis of SS.[101] TLR7 protein levels were found to be significantly higher in peripheral blood mononuclear cells (PBMCs) from SS patients than healthy controls.[102] Another study documented the presence of TLR7- and TLR9-positive cells in ductal epithelial cells, epithelial islands, and lymphocytes of the parotid glands in patients affected with primary SS.[103] More details at the cellular levels were obtained from another study, showing upregulation of TLR7, but not TLR9, in IFN+ plasma dendritic cells and monocytes isolated from PBMCs from SS patients.[104] Notably, TLR7 expression levels were found positively correlated with CXCR5, CXCL13, and TNF in salivary glands of primary SS patients.[101] As age is a risk factor of SS, it is also possible that TLR7 activity is deregulated in age-associated B-cells (ABCs). ABCs arise with age, expand in ADs, and can produce autoantibodies.[105] The role of ABCs in pathogenesis of AD is not yet clear. Possibly, TLR7 is also active in ABCs likewise in other immune cell types, triggering production of autoantibodies and so exacerbating the risk of SS with aging. Altogether, these and other data[106] support an important role for *TLR7* in the etiology and progression of SS.

CXorf21 (Chromosome X open reading frame 21; also known as *TASL*) gene is located on the short arm of the X chromosome and encodes for a protein involved

in the regulation of *TLR7* and *TLR8* signaling pathways. *CXorf21* roles in SLE have been recently investigated in detail.[107] Large genome-wide association analyses identified the Xp21.2 locus (rs887368) associated with SLE in Europeans.[108] The polymorphism is most significantly associated with the disease maps in the third exon of *CXorf21*. *CXorf21* was found to be upregulated in SLE patients exhibiting disease progression compared to those with a more stable infection.[109] Functional characterization indicated that (i) *CXorf21* expression levels are regulated by IFN and that response to IFN is higher in females than males; (ii) CXORF21 protein colocalizes with *TLR7* protein, which is also implicated in SLE pathogenesis.[107] Expression of *CXorf21* is influenced by *TLR7*, and *CXorf21* disruptions impact IFN synthesis regulated by TLR7.[110,111] As *CXorf21* closely interacts with *TLR7*, it is also believed to be an SS susceptibility gene.[112] *CXorf21* escapes XCI and is more highly expressed, at both RNA and protein levels, in female immune cells compared to male immune cells.[107,110] *CXorf21* escape may also lead to greater protein levels in female SLE cells compared to healthy controls.[110] These data support a role for XCI escape in SLE pathogenesis. However, further studies are needed to assess the extent to which altered, immune cell type-specific XCI-skew also contributes modulating *CXorf21* expression levels.

IRAK1 (Interleukin-1 receptor-associated kinase), encodes for a serine/threonine kinase involved in innate immune response through TLR signaling pathways.[113] On TLR activation, IRAK1 is recruited to the receptor-signaling complex.[114] Once activated, *IRAK1* may phosphorylate *TRAF-6*, with subsequent activation of nuclear factor kappa B (NF-κB) and other pathways and expression of IL-1/6/12 cytokines. Previous studies reported multiple genetic polymorphisms in *IRAK1* associated with both adult- and childhood-onset SLE in distinct ethnic groups.[115] Another study, including nearly 16,000 case–control subjects, identified six X-linked genetic polymorphisms mapped in the region spanning *TMEM187-IRAK1-MECP2* as to be significantly associated with SLE. The most significant signal was found at rs1059702, whose risk allele induces amino acid change in IRAK1.[116] The association of *IRAK1* with SLE was supported by other, similar analyses.[117] Interestingly, in a mouse model of SLE, IRAK1 deficiency was able to restore normal phenotype, with ablation of autoantibodies.[115] *IRAK1* is also involved in the pathogenesis of SS. A representative study highlighting *IRAK1* in SS was conducted by Zilahi *et al.*[118] They found that while *TRAF6* is overexpressed, *IRAK1* gene expression in peripheral mononuclear cells of SS patients was lower than healthy controls. In parallel, miR-146a/b, which may target both *TRAF6* and *IRAK1* was overexpressed in SS patients compared to healthy controls.[118] Several works have reported *IRAK1* as possibly subject to XCI.[33,119] However, *IRAK1* gene and protein expression levels in umbilical cord blood samples were found to be higher in females compared to males.[120] Furthermore, *IRAK1* expressed biallelically in memory B cells and plasmablast cells,[121] suggesting the occurrence of more complex mechanisms and involvement in sex-biased phenotypes.

CXCR3 (C-X-C motif chemokine receptor 3) is a chemokine receptor that binds IFN-inducible ligand chemokine and is activated by distinct IFN-inducible ligand chemokines, including *CXCL9*, *CXCL10* and *CXCL11*, all of which respond to IFN-γ.[122] These chemokines are upregulated in proinflammation and recruit immune cells at inflammatory tissue sites. *CXCR3* is primarily expressed CD4+ and CD8+ T cells and plays crucial roles in the regulation of Th cells response.[122,123] In SS, and likely in other ADs, *CXCR3* may promote inflammation and exacerbate disease symptoms and manifestation.[124] In SS patients, which may also exhibit dry eye-syndrome, the expression levels of *CXCR3* and its ligands *CXCL9*, *CXCL10*, and *CXCL11* are increased in ocular regions.[125] Another study reported that CXCR3 and its ligands may be highly expressed in lacrimal and salivary glands of SS patients.[126,127] A recent study assessed the expression of chemokine receptors and ligands in CCR9+ Th cells in primary SS patients and healthy controls. It was found that circulating CCR9+ Th cells exhibited higher *CXCR3* levels compared to CCR9- Th cells. Patients affected with primary SS also had lower levels of circulating memory CCR9+ CXCR3+ Th cells compared to healthy controls, suggesting T cell subset alteration in SS associated with cell migration upon inflammation and chemokine ligands overexpression.[128] *CXCR3* and ligands have also been implicated in SLE pathogenesis and lupus nephritis.[129] CXCR3+ T cells are abundant in kidneys and urine of SLE patients with acute nephritis, offering potential as biomarkers and therapeutic targets.[130]

CD40L (*CD40* ligand; also known as *CD154*) encodes for the ligand of CD40. It is a member of the tumor necrosis family. The CD40/CD40L pathway has been implicated in SS as crucially involved in both innate and humoral immune responses.[131,132] It is a protein predominantly expressed on the surface of T cells and can bind CD40 on the surface of B cells and antigen-presenting cells (APCs). CD40L expression can generally be detected across hematopoietic cells.[133] Upon interaction with CD40, B cells get activated, and the formation of germinal centers is stimulated.[134,135] These germinal centers, in turn, may induce formation of ectopic lymphoid sites in the salivary glands of SS patients. Both CD40 and CD40L were found highly expressed in SS infiltrating mononuclear cells from salivary gland tissue, presumably contributing to excess B cell activation,

production of autoantibodies, and inflammation,[131] Sera from SS patients is also rich in soluble CD40L,[136] further supporting the notion that both B and T cells are involved in SS pathogenesis. The activity of CD40 and CD40L also includes stimulation of proinflammatory cytokine production, that is, TNF-α and IFN-γ, in lymphoid cells. Collectively, this enhances the inflammatory milieu in ADs. The increased B cell activation and proliferation stimulated by the CD40/CD40L pathway could also mediate a higher risk of lymphoma, which is a distinctive trait of SS when compared to other ADs.[137] The CD40-CD40L axis is likely to be central in many autoimmune conditions, including to SLE pathogenesis.[138,139] For instance, CD40L is highly expressed in CD4+ and CD8+ T cells from SLE patients.[138,140,141] Flow cytometry assay revealed that peripheral monocytes expressing *CD40L* are significantly more frequent in SLE patients compared to healthy subject,[142] indicating the involvement of myeloid cells. CD40L could also be involved in complications from SLE. An earlier study reported that transgenic mouse models overexpressing CDL40 can also manifest glomerulonephritis following SLE-like symptoms.[143]

5. Concluding remarks

This review explores the prominent roles of the mammalian X chromosome in ADs. ADs are complex, heterogeneous disorders. Presumably, the interplay between genetic factors, hormonal influences, and environmental/lifestyle exposures ultimately impact the gender differences in immune system functioning at both cellular and organismal levels. The X chromosome is a model in medical genetics and is of particular scientific interest in the study of ADs. Being rich in immune-related genes and differentially inherited between the male and female sex, it plays crucial roles in genetic inheritance and disease predisposition. Females benefit from having two X chromosomes. X-linked genetic mutations may cause complete functional loss in males but not in heterozygous females, where a normal gene copy residing on the wild-type X-allele may still ensure partial functional dosages. However, the presence of two X chromosomes also correlates with an increased risk of developing ADs, with are generally more prevalent in females. As highlighted throughout this review, the functional dosage of X-linked immune-related factors is fundamentally influenced by the unique biology of the X chromosome itself. Central to this process are the highly regulated mechanisms of X-inactivation, skewed X-inactivation, and escape from X-inactivation. Altogether, these mechanisms contribute to modulating immune-related processes in time and space, impacting sex differences in both immune responses and risk of developing ADs. Clearly, more investigations are needed to fully characterize the influence of XCI-skew and XCI escape on the risk of ADs. On the other end, understanding the exact molecular mechanisms by which ADs impact X-chromosome biology, including XCI-skew and XCI escape, deserves further studies. While this review discusses a set of major ADs – systemic lupus erythematosus, Sjögren's syndrome, and Hashimoto's thyroiditis – along with several X-linked genes implicated in sex-biased ADs, there are numerous other sex-biased ADs, as well as other known X-linked genes with documented roles in ADs. Furthermore, future studies will likely uncover additional X-linked genes involved in ADs. In particular, genes that escape XCI are of great importance in understanding the pathogenetic processes underlying autoimmune traits, and may serve as target for translational research, including precision medicine approaches aimed at correcting altered gene dosages. A comprehensive understanding of the molecular factors underlying sexual dimorphisms in ADs is essential for developing targeted, sex-specific healthcare plans within the framework of personalized medicine. Such an approach would account for all known biological differences between the sexes, ultimately leading to more effective prevention, diagnosis, and treatment strategies.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

REVIEW ARTICLE

Revisiting Alport syndrome: Genetic background, phenotypic variability, and therapeutic approaches

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Abstract

Nearly a century has passed since Cecil A. Alport first described the triad of nephritis, hearing loss, and ocular abnormalities that would later be recognized as the second most common inherited nephropathy and a significant cause of end-stage kidney disease. Pathogenic variants in *COL4A3*, *COL4A4*, and *COL4A5* genes lead to compromised synthesis, assembly, and/or function of α3, α4, and α5 chains of type IV collagen (COL4). This disruption leads to an abnormal trimerization of COL4 into a stable network, impairing the integrity and function of glomerular, cochlear, and ocular basement membranes. The gold standard for Alport syndrome diagnosis is molecular genetic testing, which provides a non-invasive and highly specific approach. In settings with limited access to genetic testing, kidney biopsy with electron microscopy remains essential, revealing characteristic glomerular basement membrane abnormalities. Despite significant advancements in understanding its genetic and molecular basis, Alport syndrome remains a relentlessly progressive disorder, often culminating in end-stage kidney disease during early adulthood. While no disease-specific therapy exists, early initiation of renin-angiotensin-aldosterone system blockade is the cornerstone of AS management, delaying disease progression. Emerging therapies, including sodium-glucose cotransporter-2 inhibitors and mineralocorticoid receptor antagonists, are being investigated for their nephroprotective potential. In addition, recent breakthroughs in therapeutic research – including gene- and cell-based treatments – hold the potential to transform disease management. Genetic factors influence treatment response, reinforcing the need for personalized therapeutic approaches. In this review, we discuss the genetic background and phenotypic correlations of Alport syndrome, the pathophysiological mechanisms driving both renal and extrarenal manifestations, and explore diagnostic approaches and emerging strategies aimed at modifying the natural course of this disease.

Keywords: Alport syndrome; Inherited nephropathy; Glomerular basement membrane; Molecular genetic testing; Nephroprotection

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1. Introduction

Alport syndrome (AS) is a genetic disorder typically known by its' triad: Progressive kidney disease, sensorineural hearing loss, and ophthalmological abnormalities. It is currently the second most frequent inherited nephropathy and an important etiology of end-stage kidney disease (ESKD) worldwide.[1] It results from variants in genes encoding type IV collagen: *COL4A5* on the X chromosome and *COL4A3* and *COL4A4* on chromosome 2. Disease-related variants in these genes lead to compromised synthesis, assembly, and/or function of α3, α4, and α5 chains of type IV collagen (COL4). This disruption leads to an abnormal trimerization of COL4 into a stable network, impairing the integrity and function of glomerular, cochlear, and ocular basement membranes.[1-3]

AS can be transmitted in several ways: X-linked (XLAS), autosomal recessive (ARAS), autosomal dominant (ADAS), and, more recently, digenic inheritance has been recognized. XLAS arises from pathogenic variants in the *COL4A5* gene, ARAS, and ADAS by pathogenic variants in *COL4A3* or *COL4A4*, following a recessive or autosomal dominant inheritance pattern, respectively. The more recently recognized digenic inheritance pattern occurs when two different pathogenic variants are present in combination.[4]

2. Prevalence

The true prevalence of AS is unknown, with estimates varying widely depending on geographic and methodological differences. Traditionally, the combined phenotype-based prevalence of XLAS and ARAS has been reported between 1 in 5,000 and 1 in 50,000 in the United States and Europe, respectively.[1]

However, population-based genome sequencing analysis suggests a much higher prevalence of pathogenic *COL4A5* variants, with approximately 1 in 2,320 individuals carrying a potentially disease-causing variant. Similarly, heterozygous variants in *COL4A3* and *COL4A4* are even more common, affecting approximately 1 in 106 individuals.[5,6] These variants, while often asymptomatic, can manifest as hematuria or proteinuria and account for a significant proportion of previously unrecognized chronic kidney disease (CKD) cases. Homozygous or compound heterozygous variants in *COL4A3* or *COL4A4* are rarer, with an estimated prevalence of around 1 in 88,866.[5]

Moreover, the actual prevalence is likely higher when considering individuals already diagnosed with the disease and additional genetic variants not included in these analyses. The unexpectedly high frequency of predicted pathogenic *COL4A3* – *COL4A5* variants implies that genetic and environmental modifiers may play a crucial role in mitigating the clinical expression of AS, leading to considerable variability in disease manifestation.[6]

3. Genetic background and phenotypic correlation

Collagen is an essential structural protein in the extracellular matrix, with 28 distinct types in vertebrates. These are classified into fibrillar collagens (*e.g.*, collagen I), network-forming collagens (*e.g.*, collagen IV), beaded microfibril collagens (*e.g.*, collagen VI), multiplexins (*e.g.*, collagen XV and XVIII), and fibril-associated collagens with interrupted triple helices (FACITs) such as collagen VII and XVII.[7] Each type plays a specific role in various tissues, contributing to their mechanical and biochemical properties.

The collagen IV family consists of six distinct alpha chains (α1 to α6), each encoded by *COL4A1* through *COL4A6* genes, respectively. These chains assemble into three heterotrimers (α1α1α2, α3α4α5, and α5α5α6) that will ultimately create a critical network for the integrity and function of basement membranes.[8] The α1α1α2 is broadly distributed throughout the body, whereas α3α4α5 and α5α6α5 networks have a more tissue-specific expression. The α3α4α5 network is mainly found in the glomerular basement membrane (GBM) of the kidney, cochlea in the inner ear, and the lens capsule of the eye, thus leading to AS when mutated. Because the α1α1α2 network is a form of ubiquitous, broadly distributed network, mutations in *COL4A1* and *COL4A2* tend to result in more heterogenous phenotypes than in AS. This explains why *COL4A1* mutations are linked to conditions such as HANAC syndrome, where kidney involvement is generally mild, often presenting with multiple cysts or isolated hematuria, rather than the progressive nephropathy characteristic of AS.[9,10] *COLA42* mutations seem also to be involved in cerebral small vessel disease, being associated with intracerebral hemorrhage.[11]

Clinically, the hallmark triad of AS – progressive nephritis, hearing loss, and ocular anomalies – derives primarily from observations of affected males with XLAS. However, the clinical manifestations vary significantly based on inheritance pattern and the degree to which specific pathogenic variants disrupt the structure of the α-chains.[1,2]

Kidney involvement is the hallmark of AS, with persistent microscopic hematuria often serving as the earliest and most consistent finding.[1] This feature typically presents in early childhood and is frequently asymptomatic, identified only through family screening or routine urinalysis. Gross hematuria, though less common, may occur episodically,

often following upper respiratory infections.[12] Proteinuria develops as glomerular damage progresses secondarily to impaired GMB, along with hypertension and a gradual decline in renal function. The progression rate varies according to the inheritance pattern.

3.1. X-linked inheritance

Variants in the *COL4A5* gene account for approximately 80% of AS cases and, together with ARAS, are associated with the most severe phenotypic manifestations.[13] Approximately 70% of males develop ESKD by the age of 30, and 90% by the age of 40.[2,3]

In females with XLAS, X-chromosome activity results in a mosaic state, where segments of the GBM may have normal collagen α-chains or regions affected by the pathogenic *COL4A5* variant. This GBM mosaicism underlies their typically milder and more variable clinical presentations compared to males.[3,13] Initial studies failed to show a correlation between genotype mutations and clinical outcomes in females with XLAS, being historically labeled as "carriers." However, recent evidence recognizes them as part of the AS spectrum due to their potential for significant disease progression, with approximately 20% developing ESKD by the age of 60.[6,13] Factors associated with a higher likelihood of kidney failure in heterozygous females include early proteinuria, episodic gross hematuria, and hearing loss.

Sensorineural hearing loss is a common extrarenal manifestation of AS, particularly in XLAS and ARAS, where it affects up to 90% of male patients.[14] Hearing loss typically begins in the high-frequency range during childhood or adolescence and progresses to involve frequencies used in conversational speech. Although complete deafness is rare, early detection is essential to provide timely interventions such as hearing aids or cochlear implants.

Ocular disease is another characteristic feature of AS due to the expression of type IV collagen in ocular tissue, affecting the lens, cornea, and retina. It is observed in 30% – 40% of males and approximately 15% of females with XLAS.[13] Anterior lenticonus is pathognomonic for AS and is present in 20 – 50% of patients with XLAS or ARAS. This condition often requires surgical correction due to progressive loss of visual acuity. Dot-and-fleck retinopathy, another common ocular finding, is characterized by bilateral retinal granulations and is detectable through ophthalmoscopy or slit-lamp examination.[15] While this finding is diagnostic in the presence of a positive family history or ESKD, it does not typically impair vision. Other ocular abnormalities include posterior polymorphous corneal dystrophy, recurrent corneal erosions, macular holes, and subcapsular cataracts.[2,16] A full ophthalmologic examination is recommended for both XLAS and ARAS at the time of diagnosis, as well as periodic monitoring for potential ocular complications.[6]

A small subset of AS patients with contiguous deletions encompassing the 5' ends of the *COL4A5* and *COL4A6* may develop leiomyomatosis, a condition characterized by benign smooth muscle tumors.[17] These tumors may occur in the esophagus, tracheobronchial tree, or female reproductive tract, leading to symptoms such as dysphagia, respiratory distress, or clitoral hypertrophy. This rare manifestation occurs in 2 – 5% of patients with XLAS who have the specific chromosomal deletion.[18]

3.2. Autosomal recessive inheritance

Variants in the *COL4A3* or *COL4A4* genes lead to ARAS, a form of disease that presents with comparable severity in both males and females and is responsible for 10 – 15% of all AS cases.[19] Clinically, ARAS shares many characteristics similar to males with XLAS, with nearly all presenting with hematuria and proteinuria and with 60% progressing to ESKD. The median age for ESRD in ARAS is approximately 21 years, while sensorineural hearing loss typically manifests earlier, with a median onset age of 13 years.[19,20] Unlike XLAS, there are no known differences between females and males in ARAS.[20]

Nonsense mutations in *COL4A3* or *COL4A4* genes lead to the complete absence of collagen IV α3α4α5 heterotrimers in the GBM, whereas missense mutations result in structurally abnormal, but not necessarily deleterious, GBM. Studies have shown that ARAS patients lacking missense mutations experience an earlier onset of AS-related manifestations, such as kidney failure and sensorineural hearing loss.[20,21] A different study found that patients with nonsense mutations or mutations resulting in stop codons were associated with earlier onset of kidney failure.[22]

These studies suggest that both truncated mutations and the absence of missense mutations are associated with the worst renal prognosis, as in the XLAS patients. Furthermore, there are currently over 80 pathogenic variants identified in ARAS that make up for only 15% of AS patients, making it hard to obtain valid results in cohorts due to a low number of patients and requiring an additional study on this correlation.[22]

3.3. Autosomal dominant AS

ADAS exhibits significant variability in its clinical presentation. While some individuals experience isolated hematuria or only minimally symptomatic disease, others progress to kidney failure. Heterozygous variants of *COL4A3* or *COL4A4* are highly prevalent, affecting

more than 1% of many populations, making these genetic alterations remarkably common. These variants often manifest within family histories, with a 50% likelihood of inheritance from an affected parent.[2]

The influence of gender on clinical outcomes in ADAS remains ambiguous. Some studies indicate similar prognoses between males and females, while others suggest that males may experience a 5-year earlier decline in kidney function compared to females.[23] Kidney involvement is observed in approximately 92% of patients with ADAS, with the median age of progression to ESKD of 67 years.[24]

Hearing loss and ocular involvement appear to be uncommon in these patients. When present, they tend to occur at older ages, and it seems that ophthalmologic manifestations are even rarer than hearing loss.[23,24] However, these findings should be interpreted with caution since, due to the high prevalence of *COL4A3* and *COL4A4* mutations, medical attention may have predominantly focused on individuals with more severe disease phenotypes. This selection bias may result in an overestimation of the true severity and progression of ADAS in the broader population.

There is evidence suggesting that a significant percentage of carriers of *COL4A3* or *COL4A4* variants exhibit minimal to no kidney disease. One study reported that, among individuals undergoing genetic testing, fewer than 3% progressed ESKD by the age of 60.[4] Despite the absence of clearly defined risk factors, carriers are at a slightly increased risk for renal complications compared to the general population. However, these variants should not be viewed as the sole explanation for kidney disease in affected individuals. Alternative or additional diagnoses, such as IgA nephropathy, should be considered when evaluating such cases.[6]

3.4. Digenic inheritance

The widespread use of genetic testing has revealed several cases of digenic AS caused by coexisting pathogenic variants in *COL4A5* and *COL4A3* or *COL4A4* or variants in both *COL4A3* and *COL4A4*. These scenarios differ in population frequency, inheritance patterns, and clinical implications. Due to the low number of reported cases and limited follow-up, our current knowledge of the severity, progression, and inheritance of digenic AS remains preliminary, reflecting only observed clinical tendencies.[25]

This form of inheritance is non-Mendelian and can resemble both autosomal recessive and autosomal dominant patterns under different conditions. In certain scenarios, mutations in *trans* (such as those in *COL4A3* or *COL4A4*) can present with a 25% recurrence risk, aligning with autosomal recessive inheritance. However, mutations in *cis*, such as a combination of *COL4A5* with *COL4A3* or *COL4A4*, mirror autosomal dominant inheritance with a recurrence risk of 50%. However, when mutations in *COL4A5* and *COL4A3*/*4* coexist, the pattern of inheritance would not fit the typical Mendelian expectations, and a detailed family-specific risk assessment is necessary for these cases.[26]

3.4.1. COL4A5 and COL4A3/COL4A4 in men

In males with *COL4A5* mutations, additional *COL4A3* or *COL4A4* variants would not add to the clinical impact, as all heterotrimers are already abnormal. At present, there is no evidence linking hypomorphic *COL4A5* variants with worse outcomes.[25]

3.4.2. COL4A5 and COL4A3/COL4A4 in women

Women with digenic AS have a higher likelihood of inheriting the disease due to the two X chromosomes they each carry. In cases of a *COL4A5* mutation, about half of the heterotrimers are affected, increasing to 75% if combined with a *COL4A3* or *COL4A4* variant. However, the inheritance dynamics differ: For *COL4A5* variants, X-chromosome inactivation leads to the loss of collagen IV α3α4α5-heterotrimers in about half of the cells, while for *COL4A3* or *COL4A4* variants, only half of the total heterotrimers in each cell are defective.[25,27] The severity of the variant also matters. Severe *COL4A5* mutations typically result in earlier loss of heterotrimers, whereas mild mutations impact only a subset of cells or heterotrimers. These molecular mechanisms are consistent with clinical observations that the type of mutation significantly influences kidney disease prognosis.[27] As previously discussed, truncating mutations, such as nonsense mutations or those resulting in stop codons, are associated with earlier-onset kidney failure compared to missense mutations or splice-site variants. Therefore, clinical presentation can range widely.

3.4.3. Heterozygous variants in COL4A3 and COL4A4

Studies show that pathogenic variants in both *COL4A3* and *COL4A4* seem to be prevalent, occurring up to 5 times as often as the different supracited digenic disease mutations.[25] These two pathogenic variants may occur in *cis* (same chromosome) or in *trans* (opposite chromosome), resulting in distinct inheritance patterns and varying probabilities of disease occurrence across successive generations. While a single pathogenic variant in *COL4A3* or *COL4A4* leads to 50% of collagen IV α3α4α5 heterotrimers being defective, the presence of two pathogenic variants in these genes increases the proportion of defective heterotrimers to 75% in both male and female populations. Studies have shown that individuals with digenic pathogenic

variants in *COL4A3* and *COL4A4* exhibit a clinical course intermediate between ARAS and ADAS.[25] For instance, in a cohort of 32 patients with autosomal digenic disease, the median time to develop ESKD was 54 years, which was later than in ARAS but earlier than in ADAS. Regarding extrarenal manifestations, it was shown that over 25% of patients presented hearing loss – once again, with this prevalence being between those for ARAS and ADAS – and none had ocular abnormalities. Differences in the extrarenal manifestations among patients with this disease were not observed, regardless of their gender.

4. Diagnosis

The diagnosis of AS is made with the aid of molecular genetic testing, kidney, and/or skin biopsy. Figure 1 provides a recommended approach to diagnosing AS. Genetic testing is the gold-standard method for AS diagnosis, providing a non-invasive diagnostic route with results of a relatively high degree of specificity and sensitivity.[28] *COL4A3/4/5* gene testing is recommended in the following:[6]

- Young individuals, particularly females of childbearing age, who present with isolated, persistent hematuria of glomerular origin;
- Persistent hematuria and a family history of either hematuria or unexplained kidney disease in at least one first- or second-degree relative;
- Cases where kidney biopsy findings suggest AS;
- Patients with persistent hematuria and high-frequency sensorineural hearing loss and/or characteristic eye abnormalities, such as fleck retinopathy or anterior lenticonus;
- Individuals indicated for genetic testing for investigation of focal segmental glomerulosclerosis (FSGS) or kidney disease of unknown etiology.

In cases of ARAS or digenic AS, testing the parents of index individuals is important to assess the pathogenicity of the variant.[6] Access to genetic testing remains limited in many parts of the world due to disparities in health-care resources and reimbursement policies. In such cases, kidney biopsy plays a crucial role in the diagnosis of AS and in distinguishing it from other hereditary or acquired nephropathies with similar clinical features. Histopathological findings on biopsy – particularly those stemming from transmission electron microscopy (EM) – can reveal characteristic alterations of the GBM, such as thinning or lamellation. In addition, electron-dense deposits distinct from immune complexes and abnormal immunostaining for collagen IV chains can further support the diagnosis.[6]

This is particularly useful in differentiating AS with conditions with overlapping clinical features, such as MYH9-related disorders, which can also present with kidney failure and both hearing loss and ocular abnormalities.[29] However, the pattern on electron microscopy is different, as MYH9-related disorders do not exhibit the same GBM abnormalities.[30] Other important differential diagnoses include thin basement membrane nephropathy, which is characterized by diffuse GBM thinning but lacks the progressive structural disorganization seen in AS, and IgA nephropathy, where immunofluorescence findings play a key role in establishing the diagnosis.[31]

These findings, in combination with clinical manifestations as well as family history, aid in the selection of those who might benefit from genetic testing for arriving at a definite diagnosis. Skin biopsy might also be useful, particularly in settings without access to genetic testing and with an elevated clinical suspicion of XLAS. However, as the epidermal basement membrane lacks α3 and α4 collagen chains, this method is unsuitable for diagnosing ARAS or ADAS.[2] Once a molecular diagnosis of AS is established in an index case, genetic testing for that variant could be offered to adult relatives at risk of inheriting the disease. It has to be taken into account that 10 – 15% of pathogenic variants in *COL4A5*, and an unknown percentage of those in *COL4A3* and *COL4A4*, occur *de novo*. This is particularly important to consider when evaluating patients with no known family history of AS. The known prevalence for *de novo* variants is even weaker in individuals without any clinical manifestations of the disease, such as abnormal renal function, hematuria, proteinuria, hearing loss, or ocular findings.[6]

It is important to first offer testing to first-degree relatives before proceeding with testing of second-degree relatives. For couples undergoing preconception counseling, genetic testing can be offered to the partner if it may influence reproductive decisions or guide interventions.[6]

It is essential to point out that no targeted gene panel achieves complete sensitivity for detecting all genetic changes associated with AS, with the sensitivity of *COL4A3*, *COL4A4*, and *COL4A5* gene analysis in well-designed panels being estimated to exceed 85%, providing a reliable diagnostic tool for most pathogenic variants. However, if there is a negative result and the presence of a strong clinical suspicion, genome sequencing might be useful.[6]

Despite strong evidence supporting early intervention, implementing newborn screening for AS is still not part of routine clinical practice. The lack of a simple and detectable biomarker – such as an enzyme deficiency in Fabry disease or cysts on imaging in polycystic kidney disease – has hindered the incorporation of AS into routine newborn screening panels. Despite this limitation, advances in next-generation sequencing might provide a promising

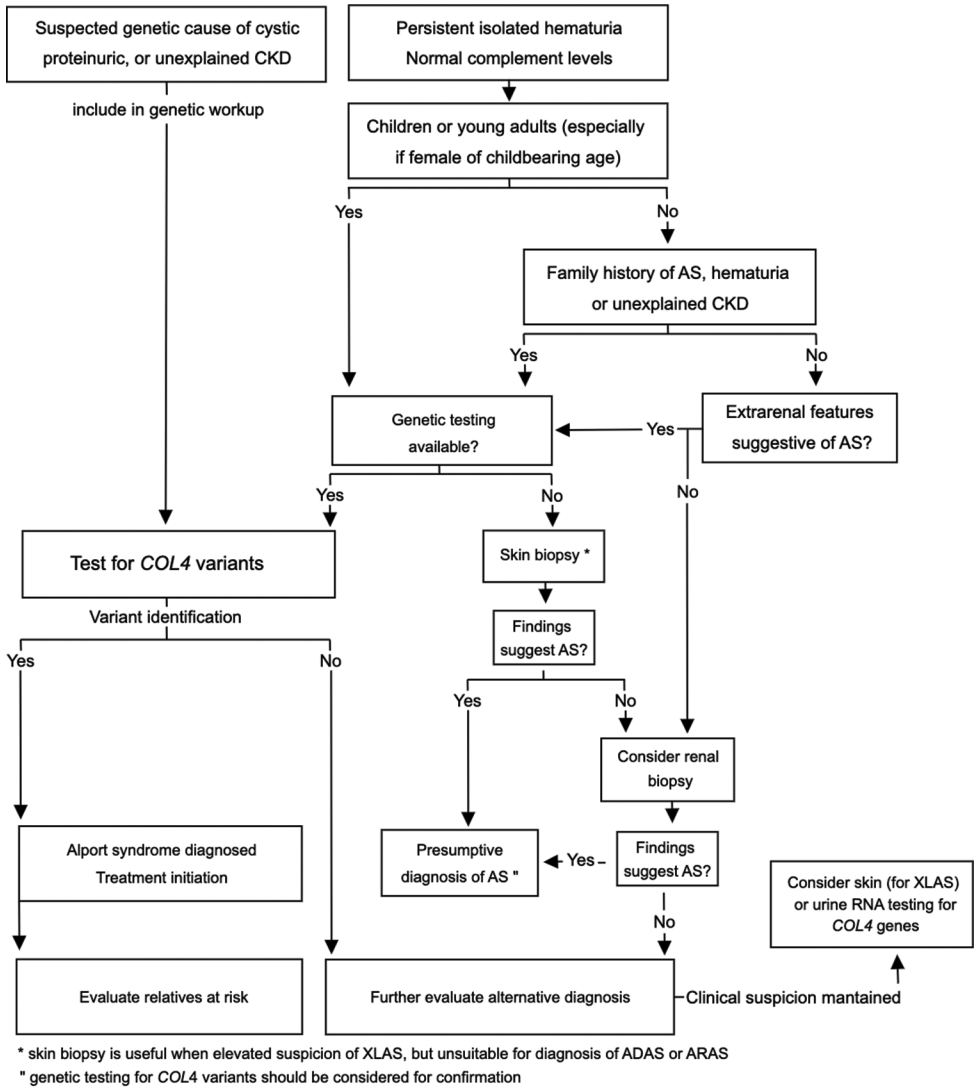

Figure 1. A recommended approach to diagnosing individuals with suspected Alport syndrome
Abbreviations: ADAS: Autosomal dominant Alport syndrome; ARAS: Autosomal recessive Alport syndrome; AS: Alport syndrome; CKD: Chronic kidney disease; XLAS: X-linked Alport syndrome.

avenue for early genetic screening, particularly in high-risk families, leading to earlier diagnosis and intervention, ultimately improving long-term renal outcomes.

5. Differential diagnosis

Clinical diagnosis can be challenging, and the assumption can only be made in the presence of a suggestive family history of XLAS and the presence of pathognomonic ocular findings. A definite diagnosis can be made by genetic testing, kidney or skin biopsy, depending on accessibility and the clinical context. A thorough personal and family history can be the key to good clinical suspicion.

The presence of isolated hematuria and proteinuria (without hearing loss or ocular manifestations) should raise suspicion for a glomerular disease. Given the earlier onset presentation, patients might be misdiagnosed with IgA nephropathy, and only a kidney biopsy can make the definitive diagnosis.[1,3] However, genetic testing has reshaped the diagnostic approach, especially in cases of hematuria and proteinuria caused by AS or other variants. Variants affecting collagen IV are shared by AS and conditions previously labeled as thin basement membrane nephropathy (TBMN), which are now recognized as part of a single spectrum of collagen IV-related diseases.[3]

The later onset and the presence of typical extrarenal manifestations of other diseases (*e.g.*, articular pain in lupus, upper respiratory tract symptoms in vasculitis) make these diagnoses less likely by default. Nonetheless, it

is mandatory to exclude other etiologies. The integration of genetic testing, including next-generation sequencing, has enhanced diagnostic accuracy for familial hematuria and proteinuria, reducing reliance on invasive procedures such as kidney biopsy. Furthermore, the reclassification of TBMN as part of the AS reinforces the risk of progression even in patients previously thought to have benign disease.[6,14] Early diagnosis and timely intervention, particularly with renin-angiotensin-aldosterone system (RAAS) blockade, have been shown to delay progression to ESKD, emphasizing the importance of accurate differentiation and proactive management in these patients.[6]

6. Therapeutic strategies

In recent years, the therapeutic options for AS have evolved with increasing focus on treatments that target the underlying mechanisms of the disease. At present, no specific therapy is approved for AS, but various options are used to delay disease progression and improve life expectancy.

6.1. Renin-angiotensin-aldosterone blockers

Although not specific for AS, RAAS blockers, including angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB), have demonstrated nephroprotective properties, including antifibrotic and anti-inflammatory effects, independent of their ability to lower blood pressure, placing this class as the cornerstone of AS treatment.[21,32] Clinical practice recommendations emphasize the early initiation of RAAS blockade in males with XLAS and both sexes with ARAS after 2 years of age. In contrast, females with XLAS and individuals with ADAS only if persistent microalbuminuria is detected.[6,33] Among the RAAS blockers, ramipril, an ACE inhibitor, is one of the most studied drugs. Studies in animal models and clinical trials, including those involving children as young as 2 years old, demonstrate its safety and efficacy. It has shown a favorable safety profile in long-term trials, such as the EARLY PRO-TECT trial.[6,34]

Due to mechanisms such as aldosterone escape, it is known that the effect of RAAS blockade diminishes throughout time. This has prompted interest in combining RAAS blockade treatment with mineralocorticoid receptor antagonists (MRA).[35] There is currently an ongoing trial – FIONA trial (NCT05196035) – recruiting pediatric patients with glomerular diseases and proteinuria, including those with AS. The aim of this trial is to assess the efficacy and the safety of finerenone, an MRA, as an adjunct therapy to RAAS blockade.[35]

Genetic factors also influence the response to therapy, with patients having milder genetic variants often experiencing better outcomes.[21,37] Nevertheless, regardless of the mutation severity, the early initiation of RAAS blockade has been linked to delayed progression to kidney failure and improved renal outcomes, emphasizing its value as a cornerstone of therapy in AS management.

6.2. Sodium-glucose cotransporter-2 inhibitors (SGLT2i)

In recent years, SGLT2i has emerged as a promising therapeutic strategy in CKD, based on their well-documented efficacy in improving cardiovascular and renal outcomes, particularly in diabetic populations.[19,38]

However, the use of SGLT2i in AS remains in its exploratory phase. Initial evidence is primarily derived from small observational studies that suggest a significant reduction in proteinuria following SGLT2i initiation. These preliminary findings indicate the potential for SGLT2i to slow renal disease progression in AS. A multicenter retrospective study involving 112 AS patients demonstrated a >30% decrease in the albumin-to-creatinine ratio (ACR) after 3 months of therapy. In addition, a modest decline in the estimated glomerular filtration rate (eGFR) of 9±12 mL/min/1.73 m^2 over approximately 1 year was observed, revealing a possible nephroprotective effect.[39]

Further evidence arises from a smaller cohort of patients with AS and FSGS, where a ~40% reduction in ACR was reported within 3 – 11 months of SGLT2i use despite the limited sample size (n = 6) and short follow-up duration.[39] While these observational studies highlight the potential role of SGLT2i in reducing proteinuria and slowing CKD progression, randomized controlled trials are urgently needed to establish their efficacy and safety conclusively. At present, an ongoing randomized controlled trial aims to evaluate the impact of SGLT2i on renal function in AS patients and potentially clarify their role in this context.[40]

The 2024 guidelines from the European Reference Network for Rare Kidney Diseases, European Renal Association, and European Society of Paediatric Nephrology recommend the addition of SGLT2i to adult patients with AS and CKD stage G2A3 or higher who continue to exhibit albuminuria despite optimized treatment with RAAS blockers. However, the guidelines do not support SGLT2i use in pediatric AS patients, nor as monotherapy in the absence of RAAS blockade, because their efficacy is maximized in combination therapy.[6]

6.3. Endothelin type A receptor (ETAR) and angiotensin II type 1 receptor inhibitors

Endothelin-1 has gained attention as a research focus due to its upregulation in AS glomeruli. In AS mouse models, sparsentan, a dual ETAR/ARB inhibitor, demonstrated

promising results, including reduced proteinuria and serum urea nitrogen and normalization of GBM morphology.[41] There are two ongoing clinical trials to evaluate endothelin receptor antagonists in glomerular diseases, including AS patients.[42,43] Preliminary results from the Phase 2 EPPIK study (NCT05003986) showed that sparsentan safely and effectively reduced proteinuria in pediatric patients with various proteinuric glomerular diseases, including AS, over the first 12 weeks of treatment. Ongoing enrollment and extended follow-up will assess long-term efficacy, **safety,** and pharmacokinetics in children.[42,44]

6.4. Lipid-lowering agents

Recent evidence highlights the significance of disrupted lipid homeostasis in glomerular cells as a key contributor and predisposing factor to CKD.[45,46] A study in model mice showed that administering hydroxypropyl-β-cyclodextrin (which binds to cholesterol) for 4 weeks to AS mice carrying *COL4A3* mutations led to reduced kidney lipid accumulation, decreased fibrosis, minimized podocyte foot process effacement in the glomeruli, and lowered urinary ACR compared to littermate controls.[47] An ongoing clinical trial (NCT05267262) that enrolled AS and FSGS patients evaluated the efficacy of R3R01, a molecule designed to enhance the activity of the ABCA1 transporter, which facilitates the removal of excess cholesterol from cells.[48]

6.5. Hydroxychloroquine (HCQ)

HCQ, an antimalarial agent and one of the oldest disease-modifying anti-rheumatic drugs, is widely recognized for its immunomodulatory properties.[49] Its mechanism of action involves disrupting Toll-like receptor signaling, which in turn reduces the activation of innate immunity. In addition, HCQ suppresses cytokine production and modulates T-cell activation by reducing CD154 expression, a molecule involved in T-cell co-stimulation.[50,51] In a retrospective study with eight pediatric patients with XLAS, all experienced reduced hematuria after 6 months of treatment with HCQ, and five of them with a concomitant decrease in proteinuria. Despite the small sample size and the short follow-up period, this study suggested the potential therapeutic benefit in XLAS patients.[52] An ongoing phase 2 clinical trial, namely the CHXLAS trial (NCT04937907), aims to further assess the efficacy and safety of this drug in AS patients.[53]

6.6. Bardoxolone methyl

Bardoxolone methyl activates the transcription factor Nrf2, playing a key role in regulating genes involved in inflammation, oxidative stress, and cellular energy metabolism.[1] Several animal studies have shown reduced kidney inflammation, fibrosis, and remodeling after therapy with bardoxolone methyl analogs, ultimately helping mitigate GFR loss over time.

The CARDINAL clinical trial was conducted to assess the effects of bardoxolone methyl in adolescent and adult patients with AS. After a 2-year study period, treatment with bardoxolone methyl led to a significant preservation of eGFR compared to the placebo group. However, a *post hoc* analysis of all available eGFR data at week 104 did not achieve statistical significance.[54,55] In addition, treatment discontinuations were more frequent in the bardoxolone methyl group (10 out of 77 patients), primarily due to protocol-specified increases in serum transaminases. Ultimately, the U.S. Food and Drug Administration declined to approve this drug, citing insufficient evidence of efficacy and safety concerns, including a potential risk of hepatotoxicity.[56]

6.7. Gene editing therapy

Genome editing therapy is a frontier concept in personalized medicine, offering the potential to treat incurable genetic diseases by correction of defective genes. At present, it is still an experimental technique for patients with AS in pre-clinical stages. Several methods have been studied, without strong evidence of a better one so far.

The exon-skipping therapy rationale is based on the use of antisense oligonucleotides to target and remove exons containing harmful mutations. Creating in-frame deletion mutations from patients with truncating mutations minimizes disease severity. In AS mouse models with *COL4A5* nonsense mutations, subcutaneous injections of these oligonucleotides partially restored COL4A5 protein expression, reduced proteinuria, and delayed kidney failure.[57]

A different study introduced a *COL4A3* transgene specifically in podocytes of COL4A3-deficient mice. This intervention preserved normal GBM structure, prevented albuminuria, and extended survival compared to untreated mice, which developed kidney failure by 8 weeks of age. Some mice presented with normal GBM even at 23 weeks of age, suggesting a lasting effect of this therapy.

The clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) system is a sophisticated genome-editing technology composed of two main components: the single-guide RNA (sgRNA) and the Cas9 endonuclease. The sgRNA is a specially designed RNA molecule that directs the Cas9 protein to a specific location in the genome by binding to a complementary DNA sequence. This high specificity ensures that the system targets only the desired genomic site. The Cas9 endonuclease then induces a double-strand break at the identified location, initiating the DNA repair process.[50,58] CRISPR/Cas9 has

been explored as a potential therapeutic tool in AS. In a study using urine-derived podocyte-lineage cells from AS patients, the CRISPR/Cas9 application achieved correction rates of 44 – 58%, reducing the proportion of indels in *COL4A3* and *COL4A5* mutations. The frequency of unintended insertions or deletions remained below 15%.[59]

Although these approaches demonstrate significant potential, their clinical application necessitates further research to overcome challenges related to safety, efficacy, and technical limitations.

7. Conclusion

AS is a complex genetic disease with a broad clinical spectrum based on its genotype, and understanding its progression is fundamental to improving clinical care. Advances in genetic testing have made it possible for an earlier diagnosis and identification of specific variants, which can help guide treatment, family planning, and even prognosis. Nowadays, RAAS blockade remains the backbone of AS treatment, although new therapy options are emerging.

SGLT2 inhibitors and experimental therapies, such as gene editing, which are more individualized. Progression depends on the genetic variant, with XLAS and ARAS causing earlier and more severe renal decline. Early diagnosis is critical for initiating effective treatment as early as possible. Continued research into AS's genetic causes, along with precision medicine, is key to improving disease management and patient quality of life.

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Conflict of interest

The authors declare they have no competing interests.

Author contributions

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Identifying regulatory variants in Indian Wilson's disease patients with missing heritability

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Abstract

Wilson's disease (WD) is a rare autosomal recessive copper metabolism disorder that primarily affects hepatic and neuronal tissues. The condition is caused by mutations in the *ATP7B* gene. Our group conducted extensive molecular genetic studies, identifying 13 clinically diagnosed Indian WD patients lacking the coding variant of *ATP7B* and 17 patients with a single mutated allele. We hypothesize that in these patients, unidentified mutations may reside in *cis*-regulatory elements of *ATP7B* or that a WD-like phenotype results from the cumulative effect of hypofunctional alleles of other key genes in the copper metabolism pathway. In this study, we employed an established bioinformatic pipeline to identify and screen *cis*-regulatory elements of *ATP7B* in WD patients with missing heritability through polymerase chain reaction sequencing. Although no pathogenic variants were identified, our analysis revealed two heterozygous single nucleotide polymorphisms (rs2181891 and rs747781) in two patients. Notably, rs2181891 showed strong regulatory potential with a RegulomeDB score of 1d. The genotype-specific expression profile for rs2181891 revealed it to be an expression quantitative trait locus for *ATP7B* in the cerebellum. In addition, the Genotype-Tissue Expression portal data suggest that the T allele of rs2181891 is associated with higher expression of *ATP7B*, making it unlikely to contribute to the WD phenotype. This novel study is the first to identify and screen *ATP7B cis*-regulatory elements in Indian WD patients with missing heritability.

Keywords: Wilson's disease; *ATP7B*; *Cis*-regulatory elements; Missing heritability

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1. Introduction

Wilson's disease (WD) is a rare autosomal recessive copper metabolism disorder characterized by abnormal copper deposition in tissues such as the liver, brain, eye, kidney, and heart.[1,2] The prevalence of WD ranges from 1 in 30,000 to 1 in 100,000 individuals.[1] WD is caused by mutations in the *ATP7B* gene, which encodes a copper-

transporting P-type adenosine triphosphatase (ATPase).[3] *ATP7B* encodes a protein with several membrane-spanning domains, an ATPase consensus sequence, a hinge domain, a phosphorylation site, and at least two putative copper-binding sites. This monomeric protein functions as a copper-transporting ATPase, exporting copper from cells, such as from the liver into the bile. Over 600 pathogenic mutations have been identified in *ATP7B*, either as homozygous or compound heterozygous variants.[4-7] The frequency of these mutations varies among different ethnicities.[8] WD patients often present with a variable age of onset and a differential spectrum of hepatic and neurological symptoms.[1,9,10] Even affected siblings within the same family can exhibit different clinical phenotypes.[11]

Other inherited disorders, such as MEDNIK syndrome,[12] Huppke–Brendel syndrome,[13] *MDR3* deficiency,[14] aceruloplasminemia,[15] congenital defects in glycosylation,[16] manganese retention disorder, and childhood cirrhosis share striking clinical features with WD.[17] Hence, molecular genetic analysis is crucial to mitigate diagnostic challenges. However, despite comprehensive genetic analyses, including sequencing of exons and splice-sites and multiplex ligation-dependent probe amplification studies to identify heterozygous indels, approximately 10 – 15% of WD patients worldwide lack mutations in both alleles of the *ATP7B* gene or have only a single mutated allele.[18,19] A recent review on the missing heritability of WD has discussed the potential locations of these uncharacterized mutations,[20] including *cis*-regulatory element(s) of *ATP7B*, where a disruptive mutation can significantly affect gene expression. Alternatively, the disease phenotype might arise from the cumulative effect of hypofunctional alleles in the other key genes of the copper metabolism pathway. In addition, the generation of reactive oxygen species and cuproptosis (a copper-dependent cell death) has been recently proposed as a molecular basis for WD endophenotypes. Increased iron deposits in the liver of WD patients may also lead to iron-related ferroptosis, contributing to phospholipid peroxidation within subcellular organelle membranes.[21] Thus, hypofunctional alleles in genes related to the cuproptosis/ferroptosis pathway may also impact the WD phenotype.

Recent advances in genomics, microscopy methodologies, and genome editing tools have unveiled the crucial role of enhancer sequences in gene expression. These enhancers interact with promoter sequences by forming chromatin loops, enabling precise spatial and temporal control of gene expression. Through these three-dimensional interactions, enhancers recruit transcriptional machinery, alter chromatin structure, and ensure finely tuned transcriptional regulation.

The advent of the Encyclopedia of DNA Elements (ENCODE) project has led to a paradigm shift in understanding the causality of genetic diseases. Variants in *cis*-regulatory elements (proximal or distal) are increasingly implicated in diseases such as cancer.[22,23] Moreover, alterations in non-coding *cis*-regulatory elements are increasingly recognized as the cause of monogenic diseases, which were once thought to be caused by mutations within coding regions. Mutations or alterations in enhancers, promoters, silencers, and insulators can perturb normal gene expression and contribute to disease pathogenesis. For instance, mutations in enhancers of the *SOX9* gene have been implicated in campomelic dysplasia,[24] a severe skeletal disorder. Similarly, mutations within the promoter of the *HBB* gene, encoding β-globin, result in reduced gene expression and are associated with certain forms of β-thalassemia.[25] In addition, dysregulation of *PAX6* due to mutations in its silencer elements is linked to aniridia, a congenital eye disorder.[26] These findings underscore the importance of *cis*-regulatory elements in maintaining gene expression fidelity and highlight their role as key contributors to the etiology of monogenic diseases.

Extensive genetic evaluation of WD patients from the eastern and western parts of India (including unpublished data from our lab) has identified a plethora of mutations in the *ATP7B* gene,[18,27-30] with p.Cys271* emerging as the most prevalent mutation.[29] However, consistent with global reports, some patients have either no detectable mutation or only a single mutated allele. Hence, we propose that mutations in the *cis*-regulatory elements of *ATP7B* may represent the uncharacterized mutations that could partially explain the missing heritability of WD. While a few studies have screened the promoter of *ATP7B*,[18,31-33] to the best of our knowledge, none has looked into the distal *cis*-elements comprehensively. Notably, Sardinian patients have been reported to have a 15-bp deletion in the promoter region of the *ATP7B* gene.[31] Wan *et al.*[34] reported two variants upstream of *ATP7B* that significantly reduced promoter activity.[34] Mukherjee *et al.*[18] also reported promoter variants in the Indian WD patients altering the expression of *ATP7B*.[18] At the same time, Chen *et al.*[35] showed that promoter mutations of *ATP7B* disrupted the binding of metal regulatory transcription factors, leading to diminished gene transcription.[35] These studies suggest that transcriptional variation of *ATP7B* could be causal to WD, as insufficient functional ATP7B proteins can disrupt cellular copper homeostasis.

Thus, our study aimed to identify the uncharacterized mutations in the *cis*-regulatory elements of *ATP7B* in a subset of WD patients with missing heritability, those

presenting with either one mutated allele or no mutation in the coding region of *ATP7B*.

2. Materials and methods

2.1. Study subjects

A total of 30 Indian WD patients, comprising 12 females and 18 males, were included in this study, selected from a larger cohort of Indian WD patients who have undergone mutation screening in the coding region of *ATP7B*.[18] The patients were diagnosed by clinicians primarily at the Bangur Institute of Neurosciences in Kolkata, National Medical College in Kolkata, and King Edward Memorial Hospital in Pune, Maharashtra, following Sternleib's criteria.[36] Key diagnostic features included the presence of Kayser-Fleischer rings, elevated 24-h urinary copper levels, low plasma ceruloplasmin levels, and abnormal hepatic and neurological features. Among these 30 clinically diagnosed WD patients, 13 lacked any coding mutations in *ATP7B*, while the remainder presented with only one mutated allele. Genomic DNA isolated from the peripheral blood of the patients was used for all genetic screenings.

2.2. Prioritization of *cis*-regulatory elements for *ATP7B* screening

The ENCODE portal provides experimentally validated information about regulatory elements (enhancers, silencers, repressors, and promoter regions) present in the genome. It integrates DNase I hypersensitive sites sequencing, chromatin immunoprecipitation sequencing, H3K4me3, H3K27ac, and CCCTC-binding factor (CTCF) data generated by ENCODE and Roadmap Epigenomics consortia. Using DNase 1 hypersensitivity, H3K4me3, H3K27ac, and CTCF signals, candidate regulatory elements were classified into enhancer-like sequences, promoters, and CTCF-bound repressors, validated across several cell types and tissues. This classification is based on data from the ENCODE database, which utilized the human genome assembly hg19. In a recent review, a total of eight candidate regulatory elements for the *ATP7B* gene were identified in the HepG2 cell line, with proximal or distal modes of regulation.[20] In this study, we selected three regulatory elements with high DNase I hypersensitivity, H3K4me3 and H3K27ac signals, and low CTCF Z-scores. Two of these regulatory elements exhibit distal regulation, while one shows proximal regulation of *ATP7B* gene expression (Table 1). The DNase I hypersensitivity indicates the open chromatin conformation, whereas H3K4Me3 and H3K27Ac denote the enhancer region. CTCF is a transcription factor generally present in a DNA element that potentially acts as a repressor.

2.3. Polymerase chain reaction (PCR) and Sanger sequencing

PCR was performed to amplify three regulatory elements of the *ATP7B* gene. Each PCR reaction was conducted in a 20 µL volume, using 80 ng of genomic DNA and GoTaq Green PCR Master Mix (Promega, India) using specific primers. The details of the primers are provided in Table S1. Following PCR amplification, bi-directional sequencing of the products was performed using the same set of primers on an ABI 3100 sequencer (Applied Biosystem, California, USA). Nucleotide changes were detected by comparing the sequences obtained in the chromatogram with the normal gene sequence using pairwise BLAST.

2.4. *In-silico* analysis of the variants

The regulatory potential of the identified variants was validated using RegulomeDB, a variant annotation software that identifies DNA features and regulatory elements in non-coding regions of the human genome. Users can input variant IDs, Browser Extensible Data files, Variant Call Format files, or General Feature Format3 files, and RegulomeDB returns a score assessing the regulatory potential of each variant. The data supporting the inference by data type and cell type can also be obtained. Accordingly, each variant we identified was assigned a score based on its expression quantitative trait locus (eQTL), transcription factor binding, DNase I hypersensitivity, and DNase I footprinting data. RegulomeDB assigns scores ranging from 1 to 6 to variants based on their regulatory potential.[37] In addition, the variants were tested for genotype-specific expression patterns and eQTL scores in tissues from the Genotype-Tissue Expression (GTex) portal.[38] The GTEx portal is a comprehensive public resource

Table 1. Regulatory elements of *ATP7B* prioritized from the Encyclopedia of DNA Elements database

Genomic coordinates	Cell type	Distance from *ATP7B* transcription start site (bp)	Length of *cis*-regulatory element (bp)	Type of regulation
chr13: 52,572,128 – 52,572,425	HepG2	13,122	297	Distal
chr13: 52,552,999 – 52,553,472	HepG2	3,695	473	Distal
chr13: 52,585,439 – 52,585,709	HepG2	0[a]	270	Proximal (5'UTR)

Note: [a]Partially overlapping with the first exon and 5'UTR of *ATP7B*.
Abbreviations: bp: Base pair; HepG2: Human liver cancer cell line; UTR: Untranslated region.

that provides valuable data on tissue- and cell-specific gene expression and regulation across different individuals, developmental stages, and species.

2.5. Statistical analysis

The age of onset, serum ceruloplasmin levels, and 24-h urinary copper levels were compared between the WD patients with no coding mutation, a single mutated allele, and both mutations of *ATP7B* using an unpaired Student's *t*-test. $P < 0.05$ were considered statistically significant.

3. Results and discussions

3.1. Comparison of clinical features of WD patients

WD patients often exhibit overlapping clinical symptoms with other early-onset diseases, complicating diagnosis for clinicians. We hypothesized that hypomorphic alleles in key copper-metabolizing genes other than *ATP7B* may contribute to the WD phenotype, potentially resulting in atypical or milder symptoms compared to patients with two *ATP7B* mutated alleles. Thus, we compared the clinical features and the age of onset between three groups of patients: those with (i) no *ATP7B* mutations, (ii) a single mutated *ATP7B* allele, and (iii) mutations in both alleles. However, no significant differences were observed between the groups when comparing the age of onset, serum ceruloplasmin levels, and 24-h urinary copper levels using unpaired Student's *t*-test with Welch's correction (Figure 1). The clinical features of all the patients are detailed in Tables S2 and S3.

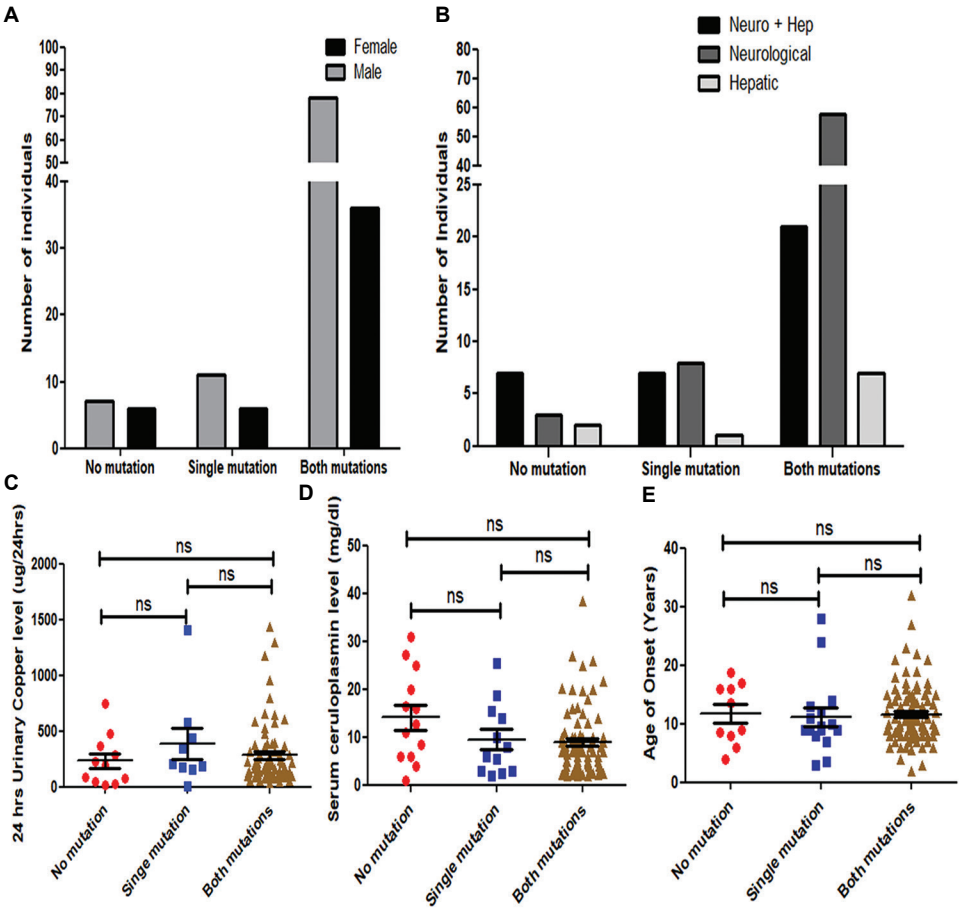

Figure 1. Comparison of clinical parameters between Wilson's disease patients with no mutation, single allelic mutation, and both coding mutations in *ATP7B*. (A) Number of males and females among WD patients. (B) Number of individuals with neurological, hepatic, and neurological and hepatic symptoms. (C) Comparison of 24-h urinary copper level (μg/24 h) between the groups using unpaired Student's *t*-test. (D) Comparison of serum ceruloplasmin levels (mg/dL) between the groups using unpaired Student's *t*-test with Welch's correction. (E) Comparison of age at the onset of symptoms between the groups of WD patients using unpaired Student's *t*-test with Welch's correction. $P<0.05$ was considered statistically significant. Analysis was performed using available clinical information.
Abbreviations: Neuro + Hep: Neurological with hepatic symptoms; ns: Not significant; WD: Wilson's disease.

3.2. Screening of *cis*-regulatory elements of *ATP7B* among Indian WD patients

We selected three non-coding *cis*-regulatory elements for the *ATP7B* gene from the list of eight previously identified elements[20] to screen in patients with either one or no mutations in *ATP7B* (Table 1). Two of these regulatory elements exhibit a distal mode of gene regulation, located 13.12 kb and 3.6 kb upstream of the transcription start site of *ATP7B*. The third regulatory element is in the 5' untranslated region of *ATP7B*. The distal regulators have high DNase I hypersensitivity and H3K27Ac marks, whereas the third element has low DNase I hypersensitivity and high H3K27Ac and H3K4me3 marks (Table 1). RNA sequence data from the ENCODE database reveal the expression of *ATP7B* in hepatic and neuronal tissues, as expected. The DNase I hypersensitivity at the selected regions correlates positively with *ATP7B* expression, as indicated by data from the Regulatory Element Database. Sequencing these three regions in the above-mentioned WD patients did not reveal any novel variants. However, we identified two heterozygous polymorphisms, rs2181891 (G/T) and rs747781 (C/T), in two WD patients – one with no mutation in *ATP7B* and another containing a single mutated allele in *ATP7B* (Figure 2A). These single nucleotide polymorphisms (SNPs) have minor allele (T) frequencies of 0.378 and 0.395, respectively, in the Bengali population in Bangladesh population, according to the 1,000 Genome Browser. They are in linkage disequilibrium with an r^2LD value of 0.96, as per the HaploReg v4.1 database.[39] The regulatory potential of these SNPs was assessed using RegulomeDB,[37] which categorizes regulatory potential using a scoring scheme ranging from 1 to 6, with lower values indicating higher regulatory potential. The rs2181891 (G/T) polymorphism shows a score of 1d, indicating its location within an eQTL, DNase I hypersensitive site, and transcription factor binding region. Notably, its eQTL effect is pronounced only in brain cerebellum tissue. Violin plots[40] for the normalized genotype-specific expression profile of *ATP7B* show that the GG genotype presents the least expression while the TT genotype shows the highest expression (Figure 3). Nonetheless, the genotype-specific expression pattern was not distinct in other neuronal and hepatic tissues. The detailed information on the SNPs identified among Indian WD patients with no mutation and single allele coding mutation identified in ATP7B is depicted in Table 2.

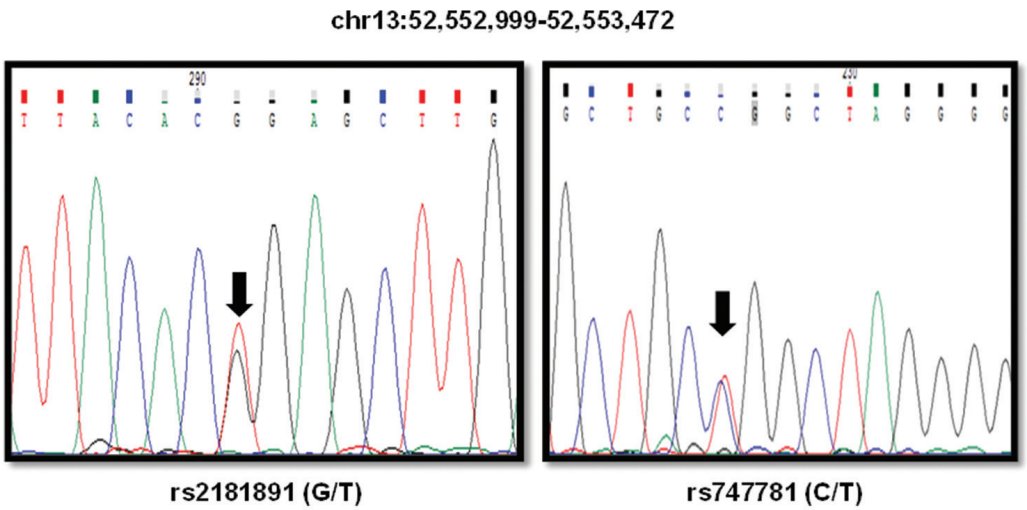

Figure 2. Chromatograms of single nucleotide variants. Single nucleotide variations (A) rs2181891 and (B) rs747781 (indicated by black arrows) identified in the *cis*-regulatory element of the *ATP7B* gene (chr13: 52,552,999 – 52,553,472).

Table 2. Single nucleotide variants identified among Indian Wilson's disease patients

Nucleotide change	Chromosomal location	Gene	No. of variant alleles identified	rsID	RegulomeDB score	eQTL
g. 52553358G >T	chr13:52,552,999–52,553,472	*ATP7B*	2/60	rs2181891	1d	Brain cerebellum
g. 52553102C >T	chr13:52,552,999–52,553,472	*ATP7B*	2/60	rs747781	4	N/A

Abbreviations: eQTL: Expression quantitative trait loci; N/A: Not available.

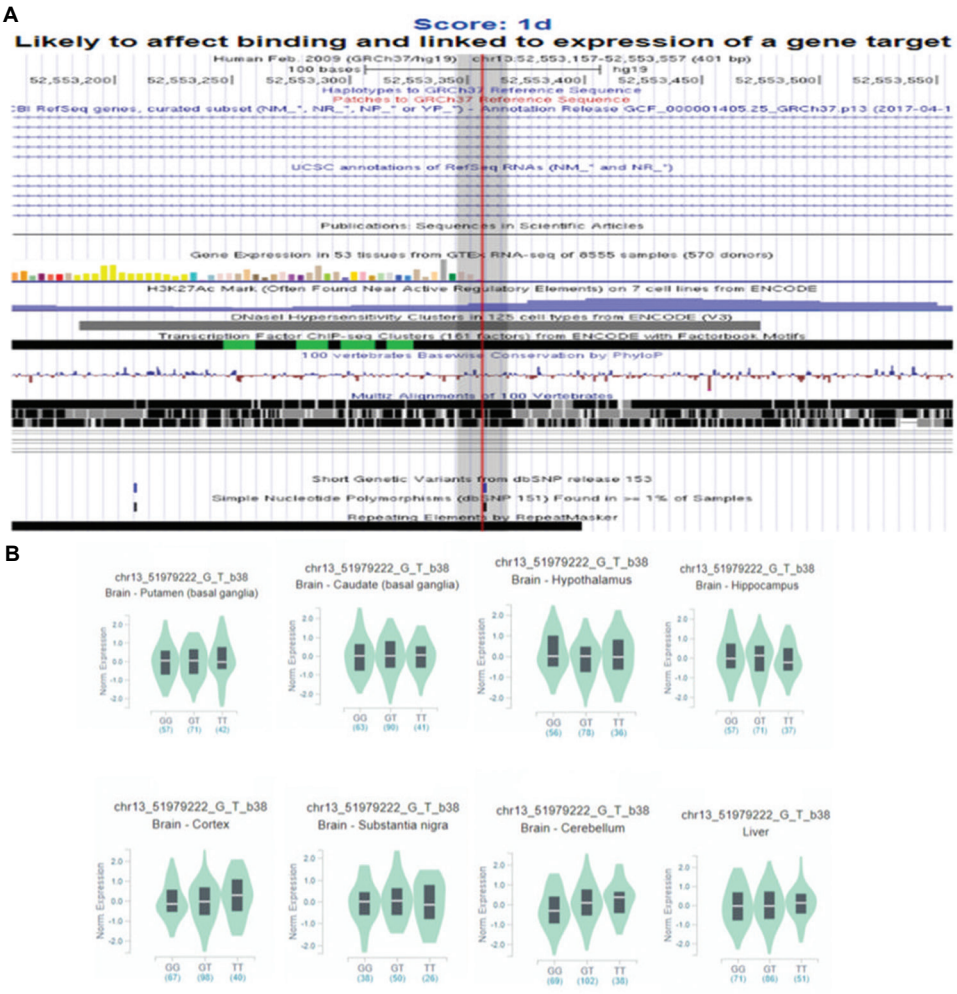

Figure 3. Allele-specific transcription factor binding alteration and gene expression of rs2181891. (A) Interpretation of RegulomeDB score from UCSC genome browser. The red line signifies the locus of the SNP. It is located within a DNase I Hypersensitive site containing an H3K27Ac mark, indicating an enhancer region. The SNP likely affects the transcription factor binding. The regulatory potential of this SNP has been validated in the GM12878 lymphoblastoid cell line. (B) The genotype-specific expression of rs2181891 was validated in hepatic and neuronal tissues from the GTEx portal. The rs2181891 (T/G) polymorphism acts as an eQTL in brain cerebellum tissue.
Abbreviations: eQTL: Expression quantitative trait loci; GTEx: Genotype-Tissue Expression; Norm.: Normalized; SNP: Single nucleotide polymorphism; UCSC: University of California, Santa Cruz.

4. Conclusion

In this study, no potential pathogenic variant in the *cis*-regulatory elements of *ATP7B* that could explain the uncharacterized mutations in the Indian WD patients was identified. However, a potentially regulatory SNP, rs2181891, was identified as heterozygous in two WD patients. Although this G/T polymorphism shows strong regulatory potential, as indicated by a RegulomeDB score of 1d, the eQTL effects of this SNP were observed only in cerebellum tissue. According to GTEx data, the variant allele (T) is associated with higher expression of *ATP7B*, which is unlikely to contribute to WD.

Our analysis was limited to screening only three *cis*-regulatory elements for *ATP7B*. As noted in our recent review, screening additional *cis*-regulatory elements and deep intronic regions of *ATP7B* may provide insights into identifying the mutations responsible for WD. Whole exome sequencing can be conducted to uncover the missing heritability for WD. Therefore, a comprehensive strategy beyond screening the *ATP7B* coding region is necessary to identify the causal variants for WD and resolve the paradox of missing heritability associated with the disease.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

The study protocols complied with the Declaration of Helsinki. In line with ICMR guidelines, we obtained institutional ethics clearance (reference number: 07/ST/20-21/1781). All participants provided informed written consent, except in the cases of minors, where their parents signed the consent form.

Consent for publication

The patients provided consent for their genetic analysis results to be published, despite being anonymous.

Availability of data

Raw data will be available on request.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Expression of MXRA7 and its prognostic significance in human bladder cancer

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Abstract

MXRA7, a gene associated with matrix remodeling, exhibits diverse expression profiles across various cancers, including bladder cancer (BLCA). Previous studies have linked elevated *MXRA7* levels to poor clinical outcomes in multiple cancer types, although its precise biological role remains unclear. In this study, bioinformatic analyses were conducted using the Cancer Genome Atlas (TCGA) data to explore *MXRA7* expression levels in BLCA. Database for annotation, visualization, and integrated discovery enrichment analysis was then employed to identify pathways associated with differentially expressed genes (DEGs) between the high expression (*MXRA7*-H) and low expression (*MXRA7*-L) groups. A least absolute shrinkage and selection operator regression model was applied to *MXRA7* and the DEGs in BLCA to generate a risk score. Multifactor Cox regression analysis, conducted using statistical product and service software automatically, was performed to identify reliable prognostic factors for patient survival. The results suggested that *MXRA7* may play a role in invasion, migration, and microenvironment remodeling in BLCA. Kaplan–Meier survival analysis revealed that higher *MXRA7* expression was significantly associated with poorer survival outcomes in BLCA. Seven key factors – "Age", "*MXRA7*", "*MXRA7* expression level", "Risk score", "Tumor grade", "Cancer status", and "Clinical_N" – were identified as components of a robust predictive model, achieving an area under curve above 0.80. These findings suggest that *MXRA7* could serve as a prognostic biomarker for BLCA and may aid in the development of targeted therapeutic strategies.

Keywords: MXRA7; Bladder cancer; Prognosis; Biomarker; Bioinformatics

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1. Introduction

Matrix Remodeling Associated 7 (*MXRA7*) was first identified in 2000 through a pioneering bioinformatic study.[1,2] So far, *MXRA7* has been shown to play significant roles in various biological processes, including liver injury model,[3] psoriasis and cutaneous injury models,[4,5] as well as the differentiation of bone marrow mesenchymal stem cells.[6] From the perspective of malignancy, *MXRA7* exhibits different expression patterns across multiple tumors, including leukemia.[7-9] Research indicates that *MXRA7* is overexpressed in several cancers such as glioma with *Pten*$^{-/+}$ genotype,[10] colorectal cancer

(both adenoma-carcinoma transition and non-metastatic cases),[11,12] breast cancer basal stem cells,[13] inflammatory breast cancer,[14] male hepatocellular carcinoma,[15] HPV-negative oropharyngeal carcinoma,[16] and malignant peripheral schwannoma.[17] In contrast, some research showed down-regulation of *MXRA7* in retinoblastoma.[18] In addition, analysis of the Human Protein Atlas from the Cancer Genome Atlas data suggested that higher *MXRA7* expression was associated with poor prognosis in urothelial carcinoma.[19] Up-regulated *MXRA7* gene levels were disclosed in high-risk patients with bladder cancer (BLCA) through differential analysis.[20] While these findings hint at a significant correlation between *MXRA7* expression and certain cancers, comprehensive studies explicitly addressing *MXRA7*'s biological significance in malignancies have been limited, apart from research conducted by our team.[7-9] The current study aimed to fill this gap by specifically analyzing *MXRA7*'s role in BLCA progression and prognosis.

As a leading cause of death, cancer poses a significant threat to human health and places a substantial financial burden on society.[21] BLCA is the fourth most common malignancy among men,[22] accounting for about 5% in metastatic diseases.[23] While therapies such as immunotherapy and targeted treatments are emerging, accurately predicting the survival of BLCA patients remains a challenge for healthcare providers and a focus of research.[24] Since MXRA7 has been suspected to be correlated with tumor progression or metastasis in some cancers,[20] defining the role of MXRA7 in cancers might provide insights into patient prognosis and support more personalized treatment strategies. Therefore, it is of significance to examine and solidify the behavior of MXRA7 in cancers, including BLCA.

A pan-cancer bioinformatic analysis by our team (submitted for publication) and others[25] suggested that MXRA7 manifested differential alterations in various cancers. The current study was performed to further analyze the role of *MXRA7* in the prognosis of BLCA through expression profiling and linear models for microarray data (Limma) analysis. Differentially expressed genes (DEGs) retrieved on the basis of *MXRA7* expression difference were subjected to the database for annotation, visualization, and integrated discovery (DAVID) enrichment assay to uncover potential pathways or gene ontology (GO) terms associated with the functions of MXRA7 in BLCA. A least absolute shrinkage and selection operator (LASSO) model[26] was then established to categorize the patients into "high-risk" and "low-risk" groups. Subsequently, multivariate Cox regression analysis was utilized to identify the significant factors for the prognostic model in clinical data. Finally, nomogram was employed to evaluate the reliability of *MXRA7* as a biomarker for BLCA prognosis.

2. Methods

2.1. BLCA transcriptome profiling and Limma differential analysis

Raw data for BLCA RNA-seq datasets were obtained from TCGA (https://portal.gdc.cancer.gov/). The v22 and v33 gff3 files were downloaded from GENCODE to map GeneSymbols to ENSG_IDs. In case multiple matches occurred, the median expression value was used to generate the transformed expression profile. The median *MXRA7* expression level was used to divide the BLCA clinical samples into high expression (*MXRA7*-H) and low expression (*MXRA7*-L) groups. When a gene symbol corresponded to multiple gene IDs, the median value was taken for generating standardized gene expression data. Differential analysis was conducted using the R package "Limma" to identify DEGs in *MXRA7*-H verse *MXRA7*-L, with fold changes of 2.0 as threshold to determine the "differential" between these two groups.[27] Expression profiles were log2 transformed, followed by the "lmFit" function for multiple linear regression and the "eBayes" function for empirical Bayes moderation of standard errors. This approach facilitated the calculation of moderated t-statistics, moderated F-statistic, and log-odds of differential expression, culminating in significance assessment of gene expression differences.[27] The results were exhibited using heat map and volcano plot to clearly visualize the differences in gene expression between the high and low-expression groups.

2.2. Pathways enrichment and functional annotation analysis

Based on *MXRA7* expression in the transcriptomic datasets derived from BLCAs, a total of 230 genes were up-regulated and 63 were down-regulated, and they were used for additional functional enrichment analysis. DAVID bioinformatics in combination with Knowledgebase v2024q2 (released on July 5, 2024) was used to identify significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and GO terms.[28,29] DEGs were selected based on a $|\log2 \text{ fold change}| > 1.0$ and an adjusted p-value (false discovery rate [FDR]) <0.05. Pathways with an FDR <0.05 were considered significant. KEGG pathway analysis was specifically performed to investigate the involvement of these DEGs in cellular processes, focusing on pathways and GO terms potentially relevant to tumorigenesis and cancer progression. The enriched pathways and functional terms were further analyzed to identify key biological processes and signaling pathways that might play a role in the biology of BLCA.

2.3. Kaplan–Meier (KM) survival analysis

The analysis of clinical characteristics of BLCA patients and *MXRA7* expression level primarily involved KM survival curve analysis to evaluate progression-free survival (PFS) and disease-free interval (DFI).[30] PFS is defined as the time from diagnosis or treatment initiation to disease progression (including local tumor growth, new lesion development, or metastasis) or death.[31] PFS is commonly used in settings where patients still have measurable disease, and its assessment helps evaluate treatment efficacy by measuring how long a patient can survive without disease worsening. Conversely, DFI refers to the time from achieving complete remission after initial treatment to the first documented recurrence of the disease.[31] DFI is particularly relevant for patients who have undergone curative treatment, such as surgery or a complete response to therapy, and serves as an indicator of recurrence risk. These definitions help distinguish different aspects of patient prognosis, with PFS reflecting ongoing disease control and treatment response, while DFI assesses the risk of recurrence after remission.[31] In this study, both metrics were analyzed to determine whether *MXRA7* expression is associated with disease progression or recurrence risk, respectively, providing insight into its potential prognostic relevance. Clinical data samples derived from TCGA were used to correlate with the clinical outcomes. After excluding samples lacking *MXRA7* expression data, a total of 408 of PFS and 161 of DFI patients were analyzed for *MXRA7* expression and survival curves using log-rank test.[30]

2.4. LASSO-Cox regression analysis

The prognostic relevance of any single gene could be evaluated using the Cox method with the "survival" R package, which combined survival time, status, and gene expression levels. Subsequently, the "glmnet" R package was utilized to merge these data, applying the LASSO Cox approach for regression analysis.[32] LASSO regression is particularly useful for selecting the most relevant genes influencing the survival time of patients with BLCA, while Cox model can analyze the relationship between survival time and multiple factors. The LASSO Cox method was preferred over traditional Cox regression due to its ability to perform automatic variable selection and reduce overfitting, which is essential for handling high-dimensional transcriptomic data.[33] Unlike standard Cox regression, which includes all variables and may face multicollinearity issues, LASSO applies an L1 penalty, shrinking irrelevant coefficients to zero and retaining only the most predictive genes.[33] Alternative models, such as Random Forest and Support Vector Machines, were considered but not preferred due to their lack of direct survival time interpretation.[34] Thus, LASSO-Cox was selected as the most robust and interpretable approach for survival analysis in BLCA.

To determine the optimal model, a 10-fold cross-validation was conducted.[35] By selecting the proper Lambda value, 15 genes were initially identified from the 230 up-regulated and 63 down-regulated DEGs. Then, downstream analyses (Cox regression) were performed on the 15 genes to further evaluate their individual prognostic significance across 325 clinical samples. A risk score was calculated based on the prognostically significant genes identified. After obtaining the risk scores for each BLCA clinical patient, the performance of the LASSO-Cox model was evaluated by the receiver operating characteristic (ROC) curve, which serves to assess the accuracy of predictive models.

2.5. Multifactor Cox regression analysis and nomogram construction

The Cox proportional hazards regression analysis was performed using statistical product and service software automatically (SPSSAU) (Version 24.0) to identify factors significantly associated with the survival of BLCA patients.[36] A total of 12 factors, including "Age", "BMI", "*MXRA7*", "Risk score", "Sex", "*MXRA7* expression level", "Cancer status", "Stage", "Tumor grade", "Clinical_T", "Clinical_N", and "Clinical_M", were included as independent variables, while the dependent variable was the survival state of patients (alive or dead). These factors can be defined as follows: age (continuous variable), BMI (body mass index), sex, cancer status (indicating whether the patient currently has an active tumor: tumor-free vs. with tumor), tumor grade (low vs. high), stage (overall cancer stage), cinical_T (tumor invasion depth: T1-T4), clinical_N (lymph node involvement: N0: no nodes, N1–N3: increasing levels of nodal metastasis, NX: unknown status), clinical_M (presence [M1] or absence [M0] of distant metastasis and MX for unknown status), *MXRA7* (measured in fragments per kilobase per million mapped fragments [FPKM]), *MXRA7* expression level (a categorical variable based on median *MXRA7* expression value), and risk score (composite score from LASSO regression). Although *MXRA7* expression is a continuous variable reflecting absolute gene expression levels, and *MXRA7* expression level is a dichotomized variable for clinical stratification, both were included to assess their independent prognostic value – allowing for precise quantification while ensuring interpretability in clinical settings, with statistical validation confirming their complementary roles in survival prediction.[37] The analysis aimed to assess the impact of these independent variables on patients' survival time. The initial Cox analysis identified seven significant factors, which included "Age", "*MXRA7*", "*MXRA7* expression level", "Risk score", "Tumor grade", "Cancer status", and "Clinical_N".

For the prognostic model evaluation, the "rms" package in R was employed to amalgamate data on survival time, survival status, and the seven significant features mentioned above".[38] A Cox proportional hazards model was utilized to construct a nomogram and calibration plots, assessing the prognostic significance of these attributes in a cohort of 325 subjects. Among muscle-invasive BLCA (MIBC) cases analyzed, 305 were classified as high-grade and 20 as low-grade. Given the rarity of low-grade MIBC, the small sample size should be taken into consideration when interpreting results. The accuracy of the model was checked by comparing it against each individual factor, using methods like the concordance index (C-index) and ROC analysis.[39] In survival analysis, C-index measures the model's ability to rank a patient's survival risk. This methodological approach facilitated a comprehensive evaluation of the model's prognostic capabilities within the studied population.[39] Overall, this approach allowed for thorough assessment of the model's ability to predict survival outcomes, with a significance level set at $p<0.05$, confirming the reliability of the results.

3. Results

3.1. Limma assay revealed DEGs based on *MXRA7* gene expression levels in BLCA

After importing the processed BLCA expression profile data along with accompanying clinical information, the TCGA samples were divided according to the *MXRA7* expression level into *MXRA7* high (*MXRA7*-H) and low (*MXRA7*-L) groups. The heat map (Figure 1A) and the volcano plot (Figure 1B) were obtained by difference analysis using the "Limma" package in R. The screening conditions for differential genes were set at $p<0.05$ and FDR <0.05, identifying 230 genes as up-regulated and 63 genes as down-regulated in *MXRA7*-H group compared to the *MXRA7*-L group. Detailed variance analysis results are shown in Table S1 in Supplementary.

3.2. KEGG pathways and GO terms enriched for *MXRA7*-associated DEGs in BLCA

When the up-regulated genes ($n = 230$) and down-regulated genes ($n = 63$) in *MXRA7*-H groups were sujected to DAVID analysis with thresholds of $p<0.05$ and FDR <0.05, no significant KEGG pathways or GO terms were enriched for down-regulated genes. In contrast, 21 pathways were significantly enriched in the Editup-regulated genes (Figure 2A and Table S2 in Supplementary). Among these, the following pathways strongly suggested the potential functional roles of MXRA7, such as "Cytoskeleton in muscle cells" for cytoskeleton and cell migration, "Focal adhesion" for cell adhesion and signal transduction, "ECM-receptor interaction" for tumor microenvironment remodeling and cell invasiveness, and "PI3K-Akt signaling pathway" for cell proliferation and anti-apoptotic mechanisms that are frequently activated in BLCA. Similar impressions were obtained when DEGs were revealed to cluster at Molecular Function, Cellular Component, and Biological Process levels (Figure 2B and Table S3 in Supplementary). Additional GO terms related to the potential function of MXRA7 included "angiogenesis", "collagen fibril organization", "basement membrane", "extracellular

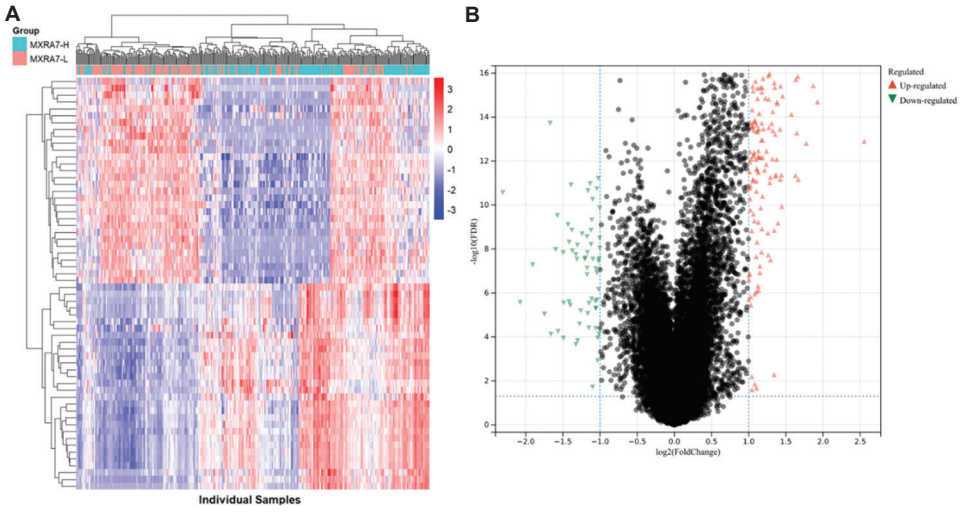

Figure 1. *MXRA7* expression-based Limma analysis in BLCA samples. (A) Heatmap of *MXRA7* expression profile (unit: FPKM), with the distance calculation method being "Euclidean" and the clustering setting as "complete". The heatmap displays the top 30 of up- and down-regulated genes according to fold changes (*MXRA7*-H/*MXRA7*-L). (B) Volcano plot showing the distribution of FDR versus fold change based on Limma differential analysis, with a cutoff fold change of 2.0.
Abbreviations: BLCA: Bladder cancer; FPKM: Fragments per kilobase per million mapped fragments; FDR: False discovery rate.

exosome", "calcium ion binding", etc. Together this, evidence suggests that MXRA7 may promote the survival, proliferation, invasion, and migration of BLCA cells through regulation of cytoskeleton or interaction with extracellular matrix.

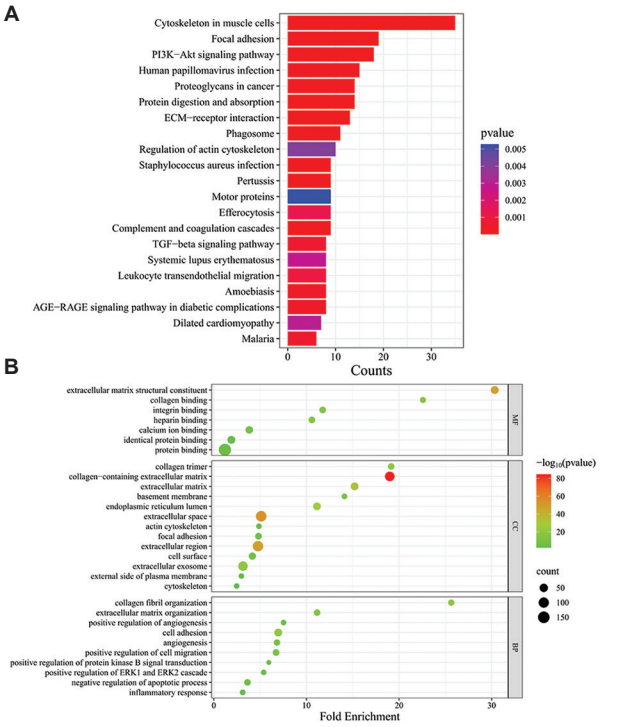

Figure 2. Functional enrichment analysis of *MXRA7*-associated genes highlighting key pathways in BLCA progression and invasiveness. (A) The KEGG crucial pathways are represented on a bar chart, with gene counts on the horizontal axis, pathway names on the vertical axis, and *p*-values indicated by color. (B) The enriched GO terms (MF, CC, BP) were depicted on a bubble diagram, with fold enrichment on the horizontal axis, pathway names on the vertical axis, point size representing gene counts, and *p*-values in -log10 indicated by color.
Abbreviations: BLCA: Bladder cancer; MF: Molecular function; CC: Cellular component; BP: Biological process; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene ontology.

3.3. Differential survival curves of BLCA patients correlated with *MXRA7* expression levels

When KM survival curves were obtained with log-rank analysis on BLCA patients with varying levels of *MXRA7*, it was noted that high *MXRA7* expression was associated with a significantly unfavorable prognosis for PFS (Figure 3A). This indicates that patients with high *MXRA7* levels are more likely to experience disease worsening or relapse, suggesting that *MXRA7* may serve as a biomarker for predicting disease progression and treatment resistance rather than recurrence risk. In contrast, no significant correlation was found between *MXRA7* expression and DFI (Figure 3B), implying that *MXRA7* expression does not strongly influence post-treatment recurrence rates in patients who have achieved complete remission.

3.4. LASSO regression analysis of DEGs

After conducting a LASSO regression analysis by integrating patients' survival data and gene expression levels (Limma) (as detailed in the Methods section), 15 genes were identified (Figure 4A), with the optimal λ approximately at 0.05 (Figure 4B), indicating the lowest model error. The heatmap showed that the expression levels of protective genes like *UPK2* decreased with risk, whereas the expression levels of risk factors like *MFAP5* increased (Figure 4C). Furthermore, as the risk score increased, the number of patient deaths increased significantly. A downstream analysis of 15 genes was then performed to further evaluate the prognostic significance of each gene in 325 clinical samples. The overall prognosis difference was significant, with key statistics including (logtest = 3.15e-08, sctest = 4.28e-10, waldtest = 3.85e-09), and C-index was 0.65. The six significant genes identified were *RRAD, UPK2, PDPN, PDLIM4, SRPX*, and *MYLK* (Figure 4D). The risk score for BLCA patients hence could be calculated using the formula: risk score = 0.009997**SRPX*-0.032084**MYLK*-0.023840**PDPN*+0.003429**PXDN*-0.062056**RRAD*-

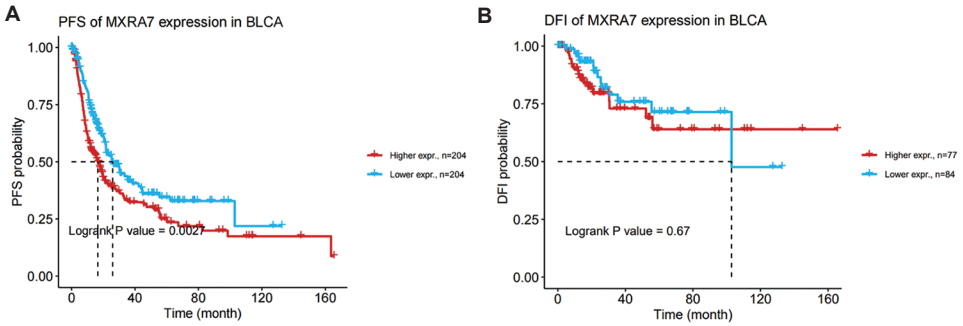

Figure 3. Kaplan–Meier survival analysis indicating high *MXRA7* expression as a prognostic risk factor for PFS in BLCA. (A) Comparison of the PFS survival curves of BLCA patients based on *MXRA7* expression levels. (B) DFI analysis in relation to *MXRA7* expression in BLCA conditions.
Abbreviations: BLCA: Bladder cancer; PFS: Progression-free survival; DFI: Disease-free interval.

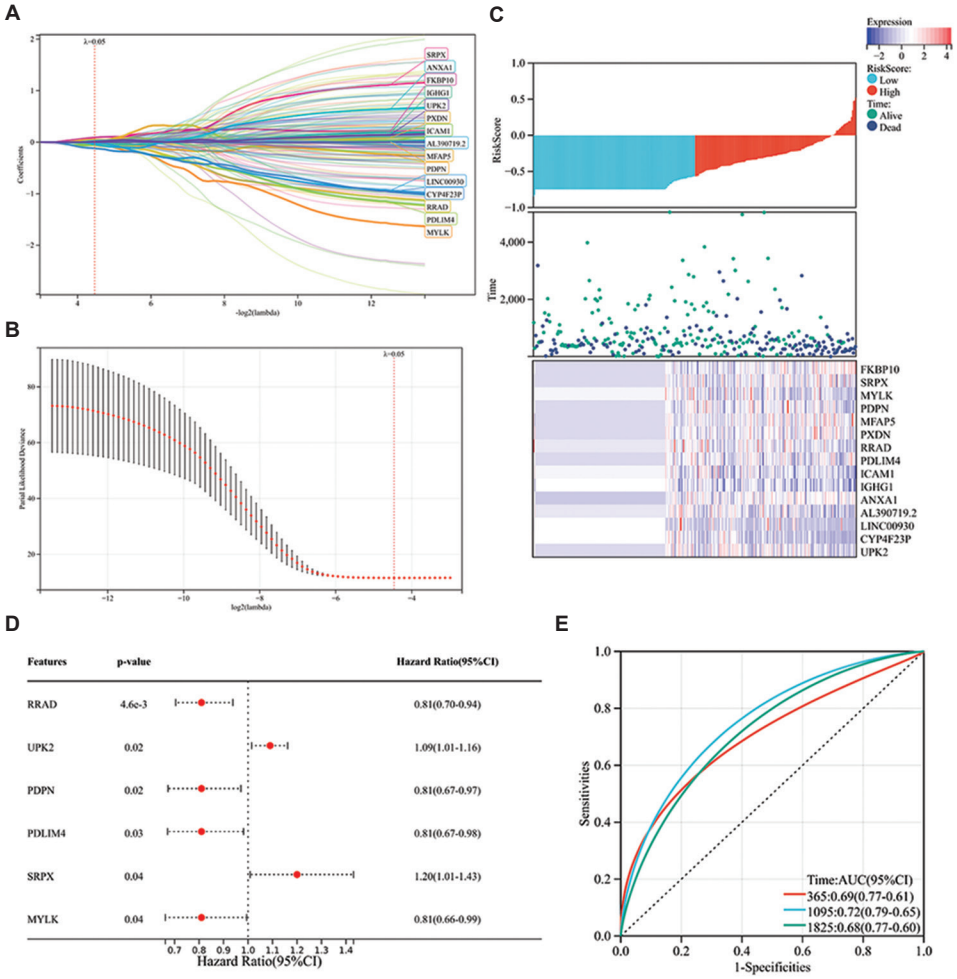

Figure 4. LASSO Cox model identifying key prognostic genes and establishing risk score for BLCA with strong predictive power. (A) Regression coefficient change curve versus -log (λ) based on LASSO-Cox analysis. (B) Partial likelihood deviance changes in LASSO regression with log (λ). (C) Relationship between risk score (top), prognostic immune gene expression (bottom), and a scatter plot of prognostic state versus time (middle). (D) Differential expression profile in gene forest map. (E) ROC curve analysis for the downstream genes is shown in Figure 4D.
Abbreviations: BLCA: Bladder cancer; LASSO: Least absolute shrinkage and selection operator; ROC: Receiver operating characteristic curve.

0.047979**PDLIM4* + 0.013591**UPK2*). In addition, ROC curve analysis of the immune prognostic model yielded area under curve (AUC) values of 0.69, 0.72, and 0.68 for 1, 3, and 5 years, respectively, indicating relatively strong predictive power (Figure 4E).

3.5. Building a nomogram based on the *MXRA7* prognostic model

The Cox multifactor analysis using SPSSAU selected 12 factors for screening, including "Age", "BMI", "*MXRA7*", "Risk score", "Sex", "*MXRA7* expression level", "Cancer status", "Stage", "Tumor grade", "Clinical_T", "Clinical_N", and "Clinical_M" as independent variables, with the existence state is represented by the patient status (alive or dead). The analysis examined the influence of independent variables on time, with results shown in Table S4 in the Supplementary. Seven factors were identified as significant: "Age", "*MXRA7*", "*MXRA7* expression level", "Risk score", "Tumor grade", "Cancer status", and "Clinical_N". Then, a Cox multifactor analysis was reconstructed for these significant factors (Table 1). The model rejected the original hypothesis (chi = 103.87, p <0.05), indicating statistical significance in model construction. Notably, the "Age", "*MXRA7*", "Risk score", "*MXRA7* expression level-Low", "Cancer Status-With tumor", "Tumor grade-Low grade", and "Clinical_N-N2" had a significant positive effect on survival risk. However, "Clinical_N-N1" and "Clinical_N-NX" did not significantly impact survival.

The nomogram combined these seven significant variables to predict whether BLCA patients could survive

Table 1. Multivariate Cox regression analysis of clinical and molecular factors in bladder cancer (*n*=325, all muscle-invasive tumors)

Term	Regression coefficient	*p*-value	HR	HR 95% CI
Age	0.03	0.01	1.03	1.01~1.04
MXRA7	0.26	0.05	1.30	0.97~1.74
Risk score	0.76	0.00	2.20	1.61~2.83
MXRA7 expression level-high	-	-	-	-
Low	0.73	0.04	1.80	1.03~3.15
Cancer status-tumor free	-	-	-	-
With tumor	0.80	0.00	2.22	1.50~3.06
Tumor grade-high grade	-	-	-	-
Low grade	0.74	0.05	1.80	0.98~3.48
Clinical_N-N0	-	-	-	-
N1	0.13	0.62	1.20	0.74~1.96
N2	0.37	0.01	1.57	1.05~2.37
N3	1.23	0.03	3.42	1.33~10.83
NX	0.65	0.06	1.91	1.01~3.59

Notes: C-index=0.60, 95% CI=0.60~0.60.
Abbreviations: CI: Confidence interval; HR: Hazard ratio.

for 1 year, 3 years, or 5 years (Figure 5A). Using this model, doctors can estimate a patient's chance of survival based on their total score. For instance, assuming that a 30-year-old BLCA patient with the following characteristics – risk score of -0.50, *MXRA7* of 8.50 (high expression), tumor grade high, N0 stage, and cancer status with tumor – would have a total score around 134 by adding the points corresponding to each feature based on the nomogram, predicting the patient having a 50% chance of survival at the 5th year. This model's accuracy was tested and showed strong reliability. The validity of the model was supported by C-index of 0.75 with 95% CI between 0.71 and 0.79 and a significant *p*-value (1.43e-35). The calibration plots showed that the model's predictions closely matched actual patient outcomes over 5 years (Figure 5B). Finally, the model was evaluated using ROC curves, which test prediction reliability over different time periods. Here, the model's AUC (a measure of accuracy) was above 0.80 for predictions at 1, 3, and 5 years, with the best accuracy at 3 years (AUC of 0.83) (Figure 5C). This means that the model is effective at identifying patients at higher risk, making it a valuable tool in personalized patient care. In summary, this model incorporates key factors such as age, *MXRA7* expression, and cancer stage to offer an accurate and reliable way for doctors to assess survival chances in BLCA patients. With its AUC above 80%, the model can be a useful tool for treatment planning and providing personalized patient prognoses.

4. Discussion

In cancer biology, the identification of biomarkers for diagnosis, treatment selection, and prognosis remains a significant challenge, and BLCA is no exception.[40,41] This study adds *MXRA7* to the growing list of potential biomarkers for BLCA, potentially through its involvement in pathways related to cell adhesion, proliferation, and anti-apoptotic mechanisms. These include the PI3K-Akt signaling pathway, ECM-receptor interaction, and focal adhesion pathways (Figure 2), all of which contribute to cancer progression by fostering an environment conducive to tumor cell survival, growth, invasion, and metastasis. Notably, *MXRA7* expression was found to be upregulated more than twofold in high-risk BLCA patients (Figure 1), consistent with findings from previous studies.[20] Furthermore, preliminary KM prognostic analysis revealed that high *MXRA7* expression was associated with poor PFS but not DFI in BLCA patients. In addition, the LASSO regression model demonstrated that *MXRA7* expression levels could effectively stratify patients into low- and high-risk groups (Figure 5). Collectively, these findings suggest that *MXRA7* is closely linked to BLCA biology and may serve as a promising target for clinical management of the disease.

As the first study to explore the role of MXRA7 in BLCA – and indeed in any solid tumor – this research provides novel insights but also has certain obvious limitations. The primary limitation is the lack of experimental validation for the bioinformatic findings using independent datasets or laboratory-based approaches. Future research should address these limitations in three key areas. First, rigorous analysis of external, independent BLCA datasets are needed to confirm the observations derived from the TCGA dataset. Datasets generated using advanced techniques, such as single-cell RNA sequencing (scRNA-seq),[42] which can differentiate cell populations within tumor tissues, would provide more robust and convincing evidence. Second, experimental validation should be conducted to confirm MXRA7's role in BLCA biology and its potential as a therapeutic target. *In vitro* studies using siRNA-mediated knockdown, CRISPR/Cas9-mediated knockout, or cDNA transfection-mediated overexpression could evaluate MXRA7's effects on cell proliferation, migration, invasion, and response to therapeutic agents, similar to previous studies conducted on leukemic cell lines in our lab.[7-9] *In vivo* validation using xenograft and metastasis models would further elucidate MXRA7's role in tumor growth and progression. Third, mechanistic investigations are needed to dissect the detailed pathways mediating MXRA7's functions. One critical question to be answered is whether *MXRA7* acts as a driver of BLCA progression or merely as a phenotypic

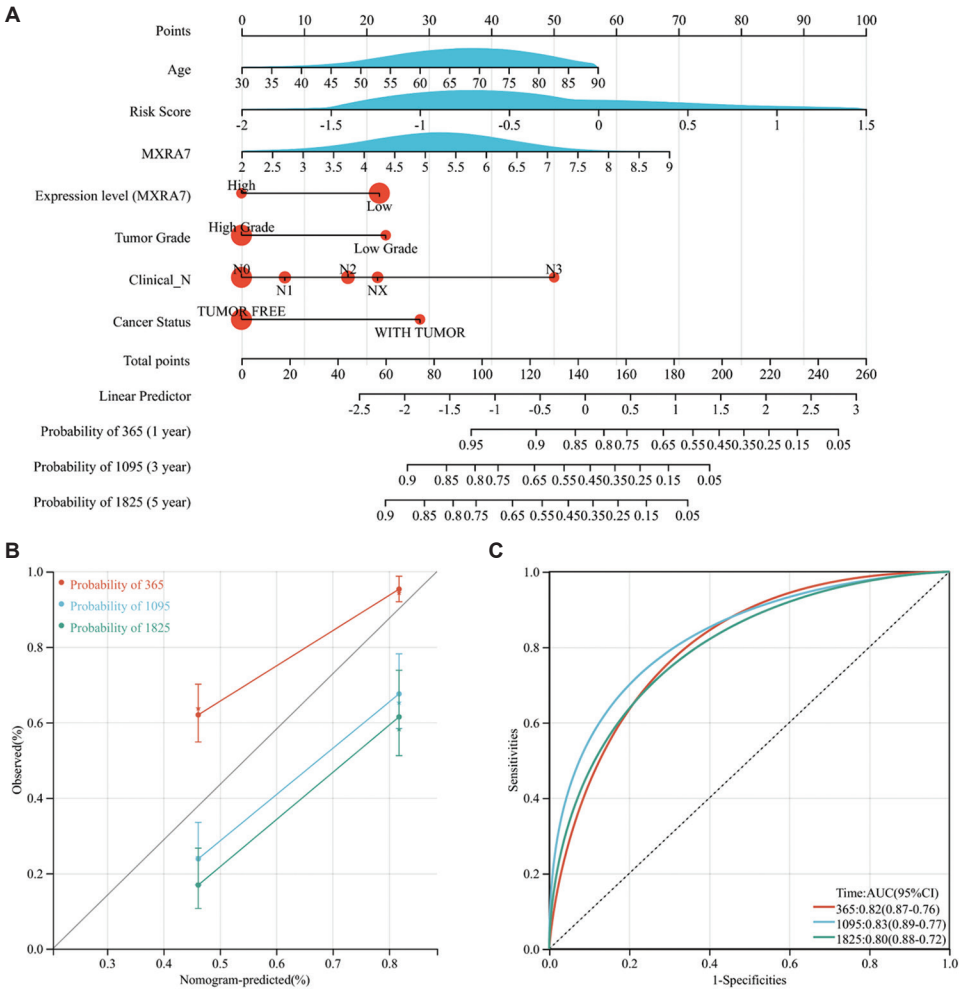

Figure 5. Establishment of a reliable prognostic model for 1-, 3-, and 5-year survival prediction in BLCA using Cox multifactor analysis and nomogram. (A) Development of a predictive nomogram for 1-, 3-, and 5-year overall survival outcomes in patients with BLCA. For each patient, individual scores corresponding to each variable are summed to yield a total point's score. This total is then mapped onto the survival probability scale at the bottom to estimate the likelihood of survival at 1, 3, and 5 years. The blue shaded area depicts the distribution of continuous variables, while the red dots represent the frequency distribution of categorical variables. (B) Verification of the predictive accuracy of the nomogram through calibration plots over 1, 3, and 5 years. (C) Analytical assessment of the nomogram through time-dependent ROC curves within the context of an immune prognostic model.
Abbreviations: BLCA: Bladder cancer; ROC: Receiver operating characteristic curve.

marker accompanying disease progression. Future studies should focus on identifying the upstream regulators and downstream effectors of *MXRA7* to better understand its role in cancer biology.

5. Conclusion

This study utilized a comprehensive bioinformatic approach to identify *MXRA7* as a significant prognostic biomarker in BLCA, with high expression levels associated with poorer survival outcomes. Functional enrichment analysis suggested that MXRA7 contributes to BLCA progression by modulating pathways related to extracellular matrix remodeling, focal adhesion, and PI3K-Akt signaling, which are critical for tumor cell survival, migration, and invasion. A risk score model developed using LASSO-Cox regression effectively stratified patients into high- and low-risk groups, while multivariate Cox analysis identified a seven-factor panel – including *MXRA7* and its expression level – as a tool for survival prediction in BLCA cohorts. These findings underscore *MXRA7*'s potential as a biomarker for prognosis and risk stratification, highlighting the need for further experimental validation to explore its mechanistic role in BLCA progression and assess its viability as a therapeutic target.

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Conflict of interest

The authors declare no competing interests.

Author contributions

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Investigation: Mingjie Chen
Methodology: Mingjie Chen, Ting Wang
Writing – original draft: Mingjie Chen, Ting Wang
Writing – review & editing: Ting Wang, Yiqiang Wang

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Not applicable.

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ORIGINAL RESEARCH ARTICLE

Differences in the expression of genes used in circadian rhythm generation in adult and pediatric gliomas

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Abstract

Altered circadian rhythms occur in several types of cancer cells. Expression of *PER2*, a core component of the circadian oscillator mechanism, and the related *PER3* gene is suppressed in adult glioblastoma (GBM). GBM cell survival depends on the activity of the core clock gene *ARNTL* that expresses BMAL1 protein, and pharmacological manipulations of BMAL1 activity are promising novel anticancer treatments. Because circadian clock gene activity in pediatric gliomas is poorly understood relative to adult GBM, we completed a meta-analysis using 19 public transcriptome datasets to evaluate expression of core clock genes and selected clock-controlled genes in adult and pediatric GBM as well as medulloblastoma (MB), pilocytic astrocytoma (PA), and ependymoma (EP) tumors. Unlike adult GBM, *PER2*, and *PER3* were not significantly downregulated in pediatric gliomas relative to non-tumor tissue. Adult GBM tissue displayed elevated expression of core clock gene *CRY1* and clock-controlled gene *NFIL3*, unlike the pediatric GBM and low-grade gliomas. The *TIMELESS* clock gene was upregulated in all glioma types except PA. The clock gene set was differentially expressed across the four standard MB subtypes in pediatric datasets and was elevated in bulk-tumor measurements and single-cell RNA sequencing results from the Group 3 and 4 subtypes. Relative to older patients, MB samples from patients under 10 years of age had six repressed core clock genes. This study found that several types of malignant pediatric brain tumors have predictable expression patterns of specific circadian clock genes and may respond to treatments using pharmacological treatments exploiting these features.

Keywords: Circadian rhythm; Glioma stem cell; Glioblastoma; Medulloblastoma; Ependymoma; Pilocytic astrocytoma; Transcriptomics

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1. Introduction

Daily rhythms in behavior and physiology are driven by molecular circadian clocks within individual cells that generate self-sustaining circadian rhythms.[1] These cellular clocks synchronize to each other and the external cycles of light and darkness, food availability, social cues, and other daily events acting on the organism. This entrainment process allows the clocks, which have intrinsic periods near 24 h, to set their phase to match predictable daily events, thereby enabling behaviors, metabolism, cell division,

DNA repair, *etc.,* to occur at the appropriate time of day. Circadian timing allows the individual to anticipate when important external events occur while optimizing the timing of internal rhythmic processes. Most notable is the sleep–wake cycle of animals in which sleep is timed, or gated, to begin and end near specific times by the master circadian clock in the hypothalamic suprachiasmatic nucleus (SCN).[2,3]

Along with circadian rhythms in the acquired and innate immune systems, the sleep cycle is fundamentally important in oncogenesis and cancer progression, mediated in part through nighttime release of the hormone melatonin.[4,5] Oncogenesis and poor patient outcomes have been attributed to chronically altered circadian rhythms of normal cells.[6] Perhaps equally important are circadian clocks within precancerous and transformed cells during cancer development. How the circadian clock impacts oncogenesis remains uncertain, but efforts are being made to understand the relationship between disruptions in the circadian timing system of the body and altered patterns of circadian gene expression in transformed cells at different stages of cancer progression. Less attention has been given to comparing clock gene regulation in very young and much older cancer patients.

Around half of protein-coding genes in mammals show a daily rhythm in gene expression depending on tissue type.[7] Many of these oscillations are generated locally by circadian clocks within the cells of tissues and organs.[3] A major transcription factor generating rhythmic expression of the clock-controlled genes (CCGs) is BMAL1, expressed by the core clock gene *ARNTL*.[8] These CCGs are regulated in part through an E-box sequence in their promoters that is activated when it binds a dimer formed by BMAL1 and an additional transcription factor CLOCK. The *PER1*, *PER2*, *CRY1*, and *CRY2* genes are directly induced through an E-box (*e.g.*, 5'-CANNTG-3'), and their protein products feed back to inhibit their own expression by interfering with the activators BMAL1 and CLOCK, thereby producing the core cycling of the circadian pacemaker. This negative feedback loop of transcription and translation works with a second transcription-translation loop that rhythmically induces *ARNTL* (at a separate phase from *PER* gene induction), generating a circadian rhythm in BMAL1 abundance by inducing members of the ROR and REV-ERB transcription factors through E-box regulation. In some cells, ARNTL2 can replace ARNTL, and NPAS2 can replace CLOCK. Pharmacological manipulation of CRY and REV-ERB proteins to suppress BMAL1 is a novel anticancer treatment.[9] Several additional proteins modify the two transcription-translation loops to control the period, amplitude, and stability of the generated circadian rhythm.

Circadian clocks are particularly relevant to gliomas because circadian rhythms in gene activity have been measured in glioma cell lines derived from human and rodent tumors, as previously reviewed.[10] Furthermore, clock gene expression in tumors formed from glioblastoma (GBM) cells implanted into the brains of live mice is synchronized with their circadian locomotor rhythms.[11] The circadian rhythms in gliomas interact with multiple metabolic processes of cancer cells that are typically altered relative to normal cells. For example, the E-box sequence in gene promoters is regulated by members of multiple transcription factor families including hypoxia-inducible factor (HIF), which responds to the low oxygen levels in tumors, c-MYC, which is a master regulator of cancer, and the SNAIL, TWIST, and ZEB proteins that are induced in metastatic cancer stem cells.[12] Much remains to be clarified concerning the molecular interactions and roles of these E-box binding regulators and circadian clocks.

Circadian rhythms are evident in the glioma stem cells (GSCs) within cancer cell cultures and in tumor spheroid cultures derived from GSCs,[9,11,13] although the non-stem cancer cells that form much of the tumor mass can also express circadian rhythms *in vitro*.[14] GSCs have properties allowing them to resist chemical and radiation cancer treatments, and can be identified through many stem cell marker proteins.[15] GSCs generally divide slowly or not at all, migrate and invade surrounding tissue, and are responsible for metastasis and, in some cases, tumor recurrence. GSCs are plentiful in specific subregions of tumors, for example, the pseudopalisading cells around necrosis and in perivascular niches of human GBM tumors.[16] Not surprisingly, GSCs are an important target of new therapeutic interventions to suppress invasive gliomas and tumor recurrence.

Early studies with animal models, particularly rodents, support the concept that disrupted circadian clocks promote cancer.[17,18] Members of the human PER gene family (*PER1*, *PER2*, and *PER3*) are underexpressed in adult GBM, a highly lethal astrocytoma with the highest WHO stage (IV), and in other cancer types,[19] although *PER3* is not considered a component of the core circadian mechanism.[20] As recently reviewed, reduction in *PER* gene expression or functional mutations of these genes is associated with more aggressive cancers, less differentiated tumor cells, and poorer patient outcomes.[21-23] Loss of *PER2* expression in response to hypoxia can also move breast cancer cells toward the epithelial-mesenchymal transition (EMT) that produces stem-like cancer cells with increased motility and invasiveness.[24-26] What has yet to be thoroughly addressed, however, is whether impaired or altered clock gene activity acts within cancer cells primarily

by impacting circadian rhythm generation or instead processes that are regulated by the clock output proteins but are not necessarily dependent on circadian timing.

Unlike adult gliomas, the expression pattern of circadian clock-related genes has not been well examined in pediatric gliomas. Here, we examined 19 datasets quantifying pediatric and human glioma transcription to describe how genes that serve in the clock mechanism and the circadian timing system are expressed. We relied on internet data portals that provide statistical tools for comparing gene expression and access to multiple adult and pediatric glioma datasets.[27] Altered expression of circadian clock genes has been characterized in adult GBM tumor cells,[28] and elevated expression of key components of the circadian clock in GBM, such as BMAL1, is recognized as an indicator of poor patient survival.[29] Although GBM is less common among pediatric cancers, we were able to compare clock gene expression in adult and pediatric GBM and in pilocytic astrocytoma (PA) of adult and pediatric patients, which is a low-grade tumor named for its morphologically distinct, hair-shaped cancer cells. PA is a common pediatric central nervous system tumor (ages 0 to 19 years) but is rare in adults.[12,30] Yet, it comprises about 1.5% of adult brain cancers.[31] Furthermore, included in this analysis were medulloblastoma (MB) and ependymoma (EP) pediatric tumors that are both low-grade but can progress to more aggressive cancers and reappear later in life. These common pediatric brain cancers differ between children and adults in their locations and molecular subtypes.[31] In the present study, clock gene expression in four previously defined MB subtypes was examined, which are also associated with different patient outcomes.[32] Expression of the clock genes in original and recurrent tumors in pediatric low-grade gliomas and GBM tumors was also compared.

2. Methods

2.1. Transcriptome data access and analysis

Publicly available collections of pediatric and adult glioma gene expression data were explored through the GlioVis data portal for visualization and analysis of brain tumor expression datasets,[27] http://gliovis.bioinfo.cnio.es/. The database was accessed until November 30, 2024. The set of circadian genes we tested was compiled from various sources and includes genes that serve in the core circadian clock mechanism. The set is listed here along with the respective proteins wherever their names differ from the genes: aryl hydrocarbon receptor nuclear translocator such as, *ARNTL* (BMAL1); *ARNTL2*; *CLOCK*; cryptochrome circadian regulator 1, *CRY1*; *CRY2*; casein kinase 1 epsilon, *CSNK1E*; neuronal PAS domain protein 2, *NPAS2*; nuclear receptor subfamily 1 group D member 1, *NR1D1* (Rev-erb-alpha); *NR1D2* (Rev-erb-beta); period 1, *PER1*; *PER2*; *PER3*; RAR related orphan receptor A, *RORA*; *RORB*, *TIMELESS*. In addition, ancillary genes were included whose proteins modulate the amplitude and stability of the rhythm[33]: Basic helix-loop-helix family member e41, *BHLHE41* (DEC2) or are major components of the clock's output pathway: D-box binding PAR bZIP transcription factor, *DBP*; hepatic leukemia factor, PAR bZIP family member, *HLF*; and thyrotroph embryonic factor, PAR bZIP family member, *TEF* that are controlled by BMAL1 and CLOCK[34] and nuclear factor, interleukin 3 regulated, *NFIL3*, controlled by Rev-erb-alpha.[35]

The selected circadian set of 20 genes includes the 13 genes in the gene set KEGG_CIRCADIAN_RHYTHM_MAMMAL (https://www.gsea-msigdb.org/gsea/msigdb/human/geneset/KEGG_CIRCADIAN_RHYTHM_MAMMAL), except for the exclusion of two gene family members (*CSNK1D* and *BHLHE40*). From these 20 genes, we also examined a subset of 12 core transcription factor genes that serve influential and intimate roles in the molecular mechanism of the circadian clock (*ARNTL*, *CLOCK*, *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER1*, *PER2*, *PER3*, *RORA*, *RORB*, and *TIMELESS*).

The Internet tools and sequence used to explore the activity of circadian genes in pediatric and adult gliomas:

(i) GlioVis to compare gene expression in normal brain tissue with GBM and lower grade gliomas (LGG) through microarray data derived from adult and pediatric patients
(ii) TIMER2.0 to compare with Gliovis GBM microarray results
(iii) UCSC Cell Viewer to compare clock gene expression in pediatric glioma subtypes and cell types previously identified through single-cell RNA sequencing (scRNA-seq)
(iv) Gliovis to compare the expression of the clock gene set in four established subtypes of medulloblastoma, which is the most common pediatric glioma
(v) Gene Expression Omnibus (GEO) to evaluate pediatric glioma clock gene expression according to age
(vi) Gliovis to compare recurrent with original tumor expression in datasets from ependymoma and pediatric GBM.

To maximize chances of detecting significant differences, selected datasets were restricted to ones with at least five patient samples in each group analyzed. In the first step, each usable pediatric or adult dataset accessed through GlioVis was examined to find differences in gene expression between the normal, non-tumor brain tissue,

and the glioma as determined by one-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) test. Statistical tests were provided by the GlioVis website interface.

Not all genes in the circadian gene set or all glioma types were present in each dataset examined. The glioma types evaluated here were glioblastoma multiforme (GBM), pilocytic astrocytoma (PA), ependymoma (EP), and medulloblastoma (MB) that had been previously distinguished according to their histology and other features. Pediatric datasets were selected from the 25 available in GlioVis. Datasets that lacked samples from normal tissue were excluded from this first analysis, in which we examined five pediatric datasets[30,36-38] and five adult datasets.[39-43] The Pomeroy 2002 dataset of pediatric gliomas in Gliovis was examined but excluded from the study because it did not provide results for eight of the 20 genes in the circadian gene set, and there were only four non-tumor samples.

To help validate the analysis using GlioVis, a Wilcoxon signed-rank test was performed through the TIMER2.0[44] website (http://timer.comp-genomics.org/timer/) to compare differences between the expression of the clock genes in adult GBM and non-tumor tissue. This analysis used data from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/). The effects of age on clock gene expression in MB were examined using GEO (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html), which provides the statistical tool Linear Models for Microarray Analysis (limma) to find differentially expressed genes in microarray data.

We used datasets of microarray and scRNA-seq results to compare clock gene expression in different subtypes of pediatric gliomas. Three medulloblastoma datasets explored through GlioVis were from Cavalli *et al.*,[45] Robinson *et al.*,[46] and Northcott *et al.*[47] for each member of the clock gene set, we compared four established tumor subtypes with each other in this bulk tumor data. The four MB subtypes were also examined through the University of California, Santa Cruz Cell Explorer website[48] (https://www.pneurooncellatlas.org/), which provides interactive exploration of cluster analyses and original scRNA-seq data generated by various studies. We used UCSC Cell Explorer to examine expression of the core circadian gene set in MB results from Riemondy *et al.*[49] and pediatric high-grade glioma (pHGG) results from DeSisto *et al.*[50] to compare tumor cell types. Significant differences between pairwise comparisons were determined using the Dwass-Steel-Chritchlow-Fligner test after significance was found by the Kruskal–Wallis test using SysStat v. 13.2.01 (Grafiti).

To explore whether recurrent and original pediatric tumors have significantly different expression of the clock gene set, we examined ependymoma datasets from Hoffman *et al.*[51] and Witt *et al.*[52] and one GBM dataset from Schwartzentruber *et al.*[53] in Gliovis. Datasets were excluded that combined several glioma types into the two groups being compared.

2.2. Expression index calculation

To summarize the significant differences between the tumor types and non-tumor tissue in the bulk tumor studies, a score ranging from -1 to 1 was calculated for each gene in the circadian dataset. We introduced this expression index (EI), which was calculated as $\sum d_i(1-p_i)/n$, where p is the significance by Tukey's HSD for the difference in expression of the gene in the tumor and non-tumor tissue, d is the direction of this difference (represented as 1 for positive, -1 for negative, or 0 for not significantly different), and n is the number of instances when this gene comparison was available in the datasets, ranging from 1 to 10. (EI was set to zero when n was zero.) The summation is for i = 1 to n instances. EI values were calculated for each gene of the circadian gene set in each of three categories – adult GBM, pediatric GBM, and the low-grade pediatric gliomas (not GBM). The solitary adult pilocytic astrocytoma dataset was not included in the EI calculations.

3. Results

3.1. Comparing pediatric and adult glioma clock gene expression

We examined the expression of 20 genes that either serve in the central molecular mechanism of the circadian clock or are well-established clock-controlled genes whose proteins control expressed rhythms. We evaluated this set of clock genes within published transcriptomic results from five microarray datasets derived from pediatric gliomas and five from adult gliomas. The focus was on these ten studies where expression was available in normal tissue from at least five patients in the same dataset for comparison.

For example, Figure 1 shows *PER2* and *PER3* expression levels in individual tumor samples across glioma types from one pediatric and one adult glioma dataset. As previously described,[19] the *PER2* and *PER3* genes were significantly suppressed in adult GBM relative to normal tissue (Table 1), but this difference was not present in pediatric GBM (Table 2 and Figure 2). Both pediatric and adult GBM were repressed relative to low-grade (LG) pediatric gliomas.

The adult and pediatric gliomas had very similar as well as distinctly different expression patterns depending on the members of the circadian clock gene set. *RORB* was

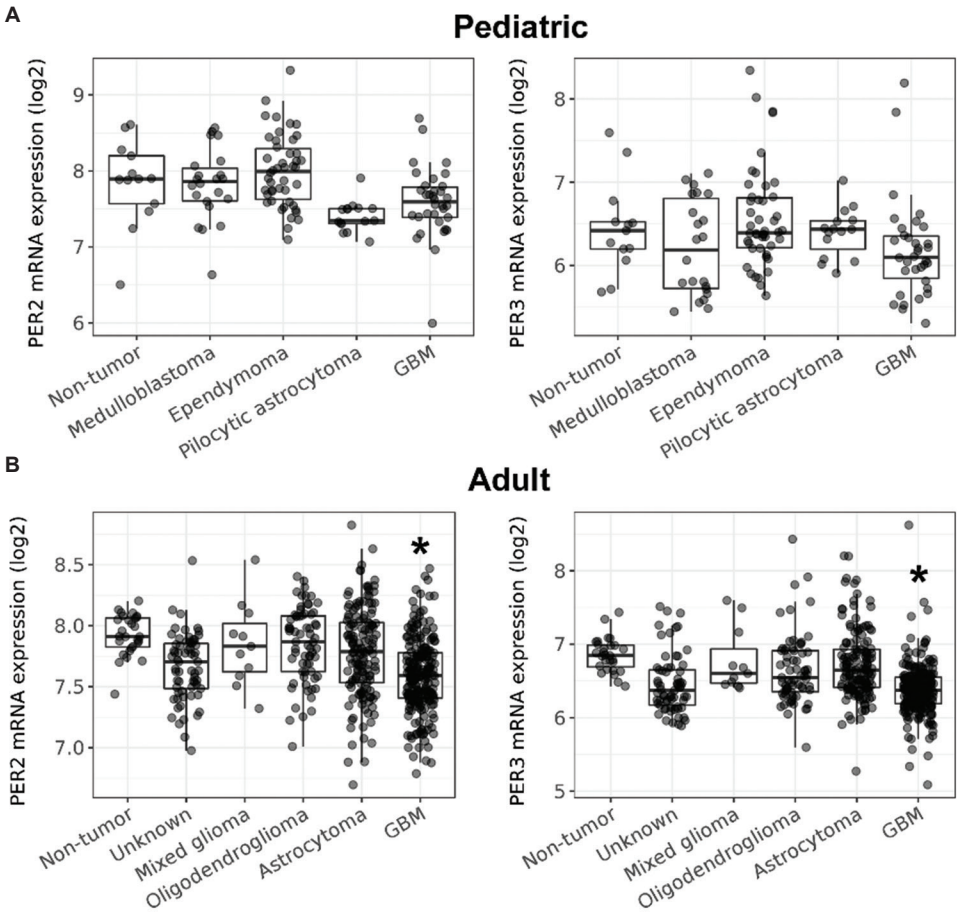


Figure 1. Gene expression in pediatric and adult gliomas. (A) PER2 and PER3 were not significantly different from non-tumor tissue in Griesinger *et al.*'s pediatric dataset.[37] Pilocytic astrocytoma-ependymoma ($p<0.001$) and GBM-ependymoma showed ($p<0.01$) significant differences in *PER2* expression, and only GBM-ependymoma showed a significant difference in PER3 expression ($p<0.05$). (B) Adult GBM *PER2* and *PER3* expression was significantly lower than in non-tumor tissue (asterisks *) in the Rembrandt dataset[40] ($p<0.001$). Other tumors showing significant differences for *PER2* were GBM-astrocytoma and GBM-oligodendroglioma ($p<0.001$); unknown-non-tumor ($p<0.01$); and oligodendroglioma-unknown ($p<0.05$). For *PER3*, the tumors included astrocytoma-unknown, GBM-oligodendroglioma, GBM-astrocytoma, unknown-non-tumor, and GBM-mixed glioma ($p<0.05$). Derived from plots and statistical tests available at the GlioVis website.[27]

significantly repressed in six of the seven GBM groups and seven of the ten LGG groups examined (Tables 1 and 2). *RORB* had a low average EI (−0.870) for datasets in all three glioma groups (adult and pediatric GBM and pediatric LG tumors) as shown in Figure 2. *CRY2* was downregulated in 15 of the 17 groups with a low average EI (−0.794). Other genes in the set that were highly downregulated in all three glioma groups were *DBP*, *HLF*, and *TEF*, which agreed with their similarly functioning proteins.[55] *TIMELESS* was unique because of its overexpression in 11 of the groups with an average EI of 0.610. The other overexpressed genes include *CRY1* and *NFIL3*, which were mostly in the adult GBM groups; *PER1* and *NR1D1* data were missing in many groups (Tables 1 and 2). Note that the EI is merely a way to summarize the expression data and shows trends in similar departures in expression from normal tissue without carrying a statistical connotation of these changes.

Pediatric GBM and LG gliomas generally showed changes in gene expression in the same direction when expression differed from normal tissue. However, the *PER* genes were not significantly repressed in pediatric gliomas, unlike in the adult GBM groups. The one low-grade adult glioma dataset examined (PA) did not show a *PER* expression pattern consistent with the adult GBM datasets (Table 2). Furthermore, unlike in the adult gliomas, *ARNTL* was downregulated in six of the 11 pediatric groups and in all pediatric glioma types but ependymal. It is unclear why the results for the clock gene set differed between the two pHGG datasets. It could have resulted from the smaller sample sizes in the Buczkowicz dataset (19 non-tumor, 35 tumor) than the Griesinger dataset (13 non-tumor, 117 tumor), which might have reduced chances of detecting a true difference. Another possibility is that the Buczkowicz dataset in GlioVis was

Table 1. Circadian clock gene expression in glioblastoma versus non-tumor brain tissue

Gene ID	Pediatric		Adult				
	GBM	GBM-type DIPG	GBM				
	Griesinger (1)	Buczkowicz (2)	Rembrandt (3)	Gravendeel (4)	TCGA (5)	Kamoun (6)	Murat (7)
ARNTL	↓**						
ARNTL2	↓*						
BHLHE41		-					
CLOCK	↓***		↓***		↓***		
CRY1			↑***	↑***	↑***	↑***	↑***
CRY2	↓***		↓***	↓***	↓***	↓***	↓***
CSNK1E			↓**			↑**	
DBP	↓***		↓***	↓**	↓***	↓**	↓***
HLF	↓***		↓***	↓***	↓***	↓***	↓***
NFIL3			↑***	↑***	↑**		
NPAS2	↓*		↓***		↓***		
NR1D1	-	↓**	-	-	-	-	-
NR1D2	↓***		↓***	↓***	↓***		↓*
PER1	-		↑***			-	
PER2			↓***		↓***		↓***
PER3			↓***	↓***		↓**	↓**
RORA							
RORB	↓***	↓*	↓***	↓***	↓*		↓*
TEF	↓***		↓***		↓***	↓***	
TIMELESS	↑***		↑***	↑***	↑***	↑***	↑***
	↓*** (p<0.001)	↓** (p<0.01)	↓* (p<0.05)	Not significantly different	↑* (p<0.05)	↑** (p<0.01)	↑*** (p<0.001)

Notes: Differences in gene expression between GBM and non-tumor tissue were determined by ANOVA and Tukey's HSD. Pediatric GBM is grouped with the GBM histological subtype of diffuse intrinsic pontine glioma (DIPG). (1) Griesinger *et al.*[37]; (2) Buczkowicz *et al.*[36]; (3) Madhavan *et al.*[40]; (4) Gravendeel *et al.*[41]; (5) Human Genome U133A array, The Cancer Genome Atlas[39]; (6) Kamoun *et al.*[42]; (7) Murat *et al.*[43]; ↓ indicates expression of this gene in the tumor tissue is significantly less than the control non-cancer (normal) tissue; ↑ indicates expression of this gene in the tumor tissue is significantly higher than the control non-cancer (normal) tissue; * indicates p<0.05; ** indicates P<0.01; *** indicates p<0.001; The colors indicate the significance of the difference (higher or lower from the non-cancer controls); - indicates data not available through GlioVis.

derived from a GBM subtype in a study of diffuse intrinsic pontine glioma (DIPG), which is a second type of pHGG along with GBM.

To validate results from the Tukey's HSD test used in Tables 1 and 2, we compared the same set of clock genes expressed in adult GBM with non-tumor tissue in the TCGA dataset but with a different online resource, TIMER2.0, that uses the Wilcoxon signed-rank test (Table 3). The significant and non-significant differences agreed with the results determined by ANOVA and HSD, except that the Wilcoxon test detected downregulation of two additional genes: *NR1D1* and *RORA*. Although *NR1D1* and *PER1* expression were reported through TIMER2.0, these genes were usually not reported in Gliovis. We suspect that this absence was attributed to the two genes being located within the sequences of *NR1D1* and *PER1*, thyroid hormone receptor alpha (*THRA*), and microRNA 6883 (*MIR6883*), respectively, interfering with the detection or reporting of the two clock genes in some datasets provided by GlioVis.

3.2. Comparing glioma subtypes and cell types

3.2.1. Pediatric glioblastoma

Recent single-cell RNA-seq (scRNA-seq) studies of pediatric gliomas provide an opportunity to compare gene expression between tumor cell types or between established tumor subtypes. We used the UCSC Cell Viewer[48] to perform these analyses and to compare scRNA-seq with bulk tumor expression results. Expression of a limited set of 12 genes that serve as transcription factors in the timing mechanism of circadian oscillators was evaluated using results provided by DeSisto *et al.*[50] They identified in their study of pHGGs three major cancer cell types that appeared to provide equally to mitotic growth

Table 2. Circadian clock gene expression in low-grade gliomas versus non-tumor brain tissue

Gene ID	Pediatric									Adult
	PA			EP			MB			PA
	GR (1)	LB (2)	GM (3)	GR (1)	GM (3)	DB (4)	GR (1)	GM (3)	DB (4)	GV (5)
ARNTL	↓**	↓*	↓*				↓***	↓***		
ARNTL2		↑*		↓***			↓***			
BHLHE41		↑***		↑**						
CLOCK										
CRY1		↓***		↑*						↑*
CRY2	↓***	↓***	↓***	↓***	↓***	↓*	↓***	↓***	↓*	
CSNK1E		↑**					↑***	↑***		
DBP	↓***	↓*	↓*	↓***	↓**	↓**	↓***	↓**	↓*	
HLF	↓***		↓***	↓***	↓**	↓**	↓***	↓**	↓*	↓**
NFIL3										
NPAS2		↑***		↓***			↓***	↓***		
NR1D1	-	-	-	-	-	-	-	-	-	-
NR1D2				↓**		↓**			↓**	
PER1	-	-	-	-	-	-	-	-	-	
PER2										
PER3						↓*			↓**	
RORA		↓**				↓**	↓*		↓***	↑**
RORB	↓***	↓***	↓***	↓***			↓***	↓***		↓***
TEF	↓**	↓*	↓***	↓***	↓**		↓**	↓***		
TIMELESS		↓**		↑**		↑**	↑***	↑***	↑***	
	↓*** (p<0.001)	↓** (p<0.01)	↓* (p<0.05)	Not significantly different	↑* (p<0.05)	↑** (p<0.01)		↑*** (p<0.001)		

Notes: Differences in expression determined by ANOVA and Tukey's HSD. (1) GR: Griesinger *et al.*[37]; (2) LB: Lambert *et al.*[30]; (3) GM: Gump *et al.*[38]; (4) DB: de Bont *et al.*[54]; (5) GV: Gravendeel *et al.*[41] The Lambert control group contains five pediatric and one adult cerebellum sample. The de Bont control group was derived from adults; ↓ indicates expression of this gene in the tumor tissue is significantly less than the control non-cancer (normal) tissue; ↑ indicates expression of this gene in the tumor tissue is significantly higher than the control non-cancer (normal) tissue; * indicates p<0.05; ** indicates P<0.01; *** indicates p<0.001; The colors indicate the significance of the difference (higher or lower from the non-cancer controls); - indicates data not available.

Abbreviations: EP: Ependymoma; MB: Medulloblastoma; PA: Pilocytic astrocytoma.

and tumor enlargement: astrocytes (AC), oligodendrocyte progenitor cells (OPCs), and mesenchymal cells (MC). They also described the MC and OPC groups as closely related according to embryonic stem cell and mitotic gene expression patterns evident through gene set enrichment analysis (GSEA). Non-malignant, immune cells of the pHGG samples were also examined with scRNA-seq and cluster analysis (Figure 3A and B). We found that *RORA* was expressed well in all three principal cancer cell types and in specific clusters of immune cells (Figure 3C and D).

We found that the entire clock gene set has widespread expression across the three principal cancer cell types (Figure 3E). On average, the genes were expressed at significantly lower levels in the OPC than in the AC (p=0.022) and MC (p=0.011) according to the Kruskal–Wallis Test (H = 10.392, p=0.006, n = 12, 2 df). Ten of the twelve clock genes showed the lowest expression in OPC. To compare malignant cells with immune cells, the average expression of each of the 12 genes across the OPC, AC, and MC cell types was found, and then these were compared with their expression in all immune cell types. Average expression of the limited clock gene set within the three malignant cell types (0.6867, ±0.1342 SD, n = 12 genes) was not significantly different from that in non-malignant, primarily immune cells of the tumor (0.7141, SD: ±0.2555 SD, n = 12) by t-test (p=0.6267) as shown in Figure 3F. In fact, the two groups were significantly correlated, as shown by Pearson correlation analysis (0.6854, p<0.001, n = 12 genes).

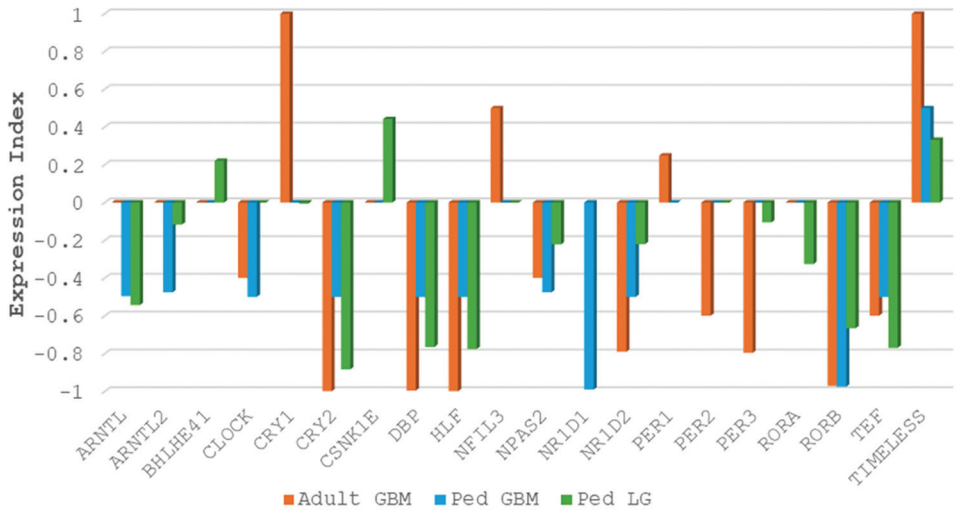


Figure 2. Comparison of the expression of the clock gene set in adult GBM, pediatric GBM, and low-grade pediatric gliomas. Consistently suppressed genes included *CRY2*, *DBP*, *HLF*, *RORB*, and *TEF* with an expression index of at least 0.5. Unlike adult GBM, pediatric gliomas show suppressed *ARNTL* and *ARNTL2* and do not have substantially suppressed *PER2* and *PER3*. Adult GBM tended to have overexpressed *CRY1* and *NFIL3*. *TIMELESS* is overexpressed in all three glioma categories. The expression index describes the combined significant differences in expression between each gene in the glioma and the corresponding non-tumor tissue. The pediatric GBM group includes two types of pHGG: GBM and a GBM-like subset of DIPG.
Abbreviations: DIPG: Diffuse intrinsic pontine glioma; GBM: Glioblastoma; pHGG: Pediatric high-grade glioma.

Table 3. Circadian clock gene expression compared between TCGA adult GBM and non-tumor tissue

Gene ID	Upregulated	Downregulated
ARNTL	0.161	
ARNTL2	0.433	
BHLHE41		0.292
CLOCK		**0.00711**
CRY1	**0.00558**	
CRY2		**0.000167**
CSNK1E		0.382
DBP		**0.000472**
HLF		**0.000278**
NFIL3	**0.0145**	
NPAS2		**0.0150**
NR1D1		**0.00108**
NR1D2		**0.000247**
PER1	**0.0876**	
PER2		**0.00182**
PER3		**0.00076**
RORA		**0.0327**
RORB		**0.0145**
TEF		**0.000167**
TIMELESS	**0.0003**	

Notes: Significant differences by the Wilcoxon signed-rank test ($p<0.05$) are shown in bold; The colors indicate the significance of the difference (higher or lower from the non-cancer controls); Data provided by TIMER2.0 (http://timer. comp-genomics.org/timer/).
Abbreviation: GBM: Glioblastoma.

3.2.2. Medulloblastoma

To compare relative expression levels of the clock gene set between four standard subtypes of medulloblastomas, we used three additional pediatric datasets and the ability of GlioVis to provide pairwise comparisons (Table 4). These subtypes consisted of WNT, enriched in wingless-type gene expression; SHH, enriched in sonic hedgehog gene expression; and the Group 3 and Group 4 subtypes that were previously known as subtypes C and D, respectively.[56]

The differences between subtypes were evaluated for each of the four subtypes by counting the number of times a clock gene was expressed in a subgroup at a significantly higher level than in another subgroup by Tukey's HSD ($p<0.05$). The resulting ranking was Group 4 > Group 3 > WNT >SHH (Figure 4). To determine which members of the circadian gene set were more highly expressed in each subtype, we used an arbitrary threshold of five counts, more than half of the nine possible counts (3 pairwise comparisons × 3 datasets). Group 4 exceeded that threshold with *CLOCK*, *CRY1*, *CRY2*, *HLF*, *NFIL3*, *NPAS2*, and *PER2*. For Group 3, the significant genes were *ARNTL* and *CRY1*, and for WNT and SHH, they were *RORA* and *CSNK1E*, respectively. *ARNTL*, *CRY1*, and *TIMELESS* were consistently highest in Groups 3 and 4 across all three datasets. Note that this analysis only considers comparisons between the four subtypes and does not indicate whether a gene is more highly expressed than average for genes in that dataset.

To compare the gene activity of the MB subtypes, we relied on scRNA-seq data and cluster analysis results provided

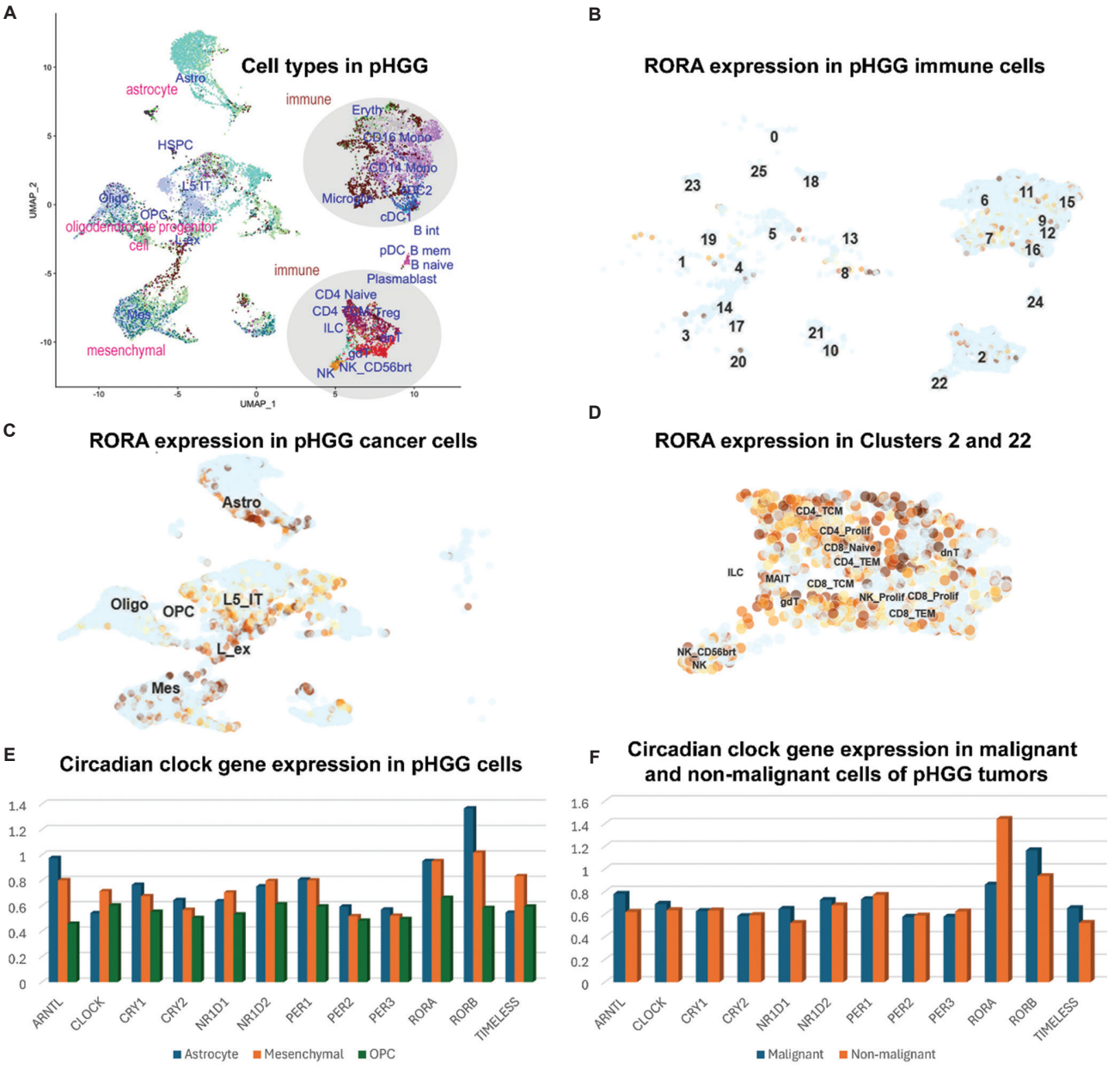
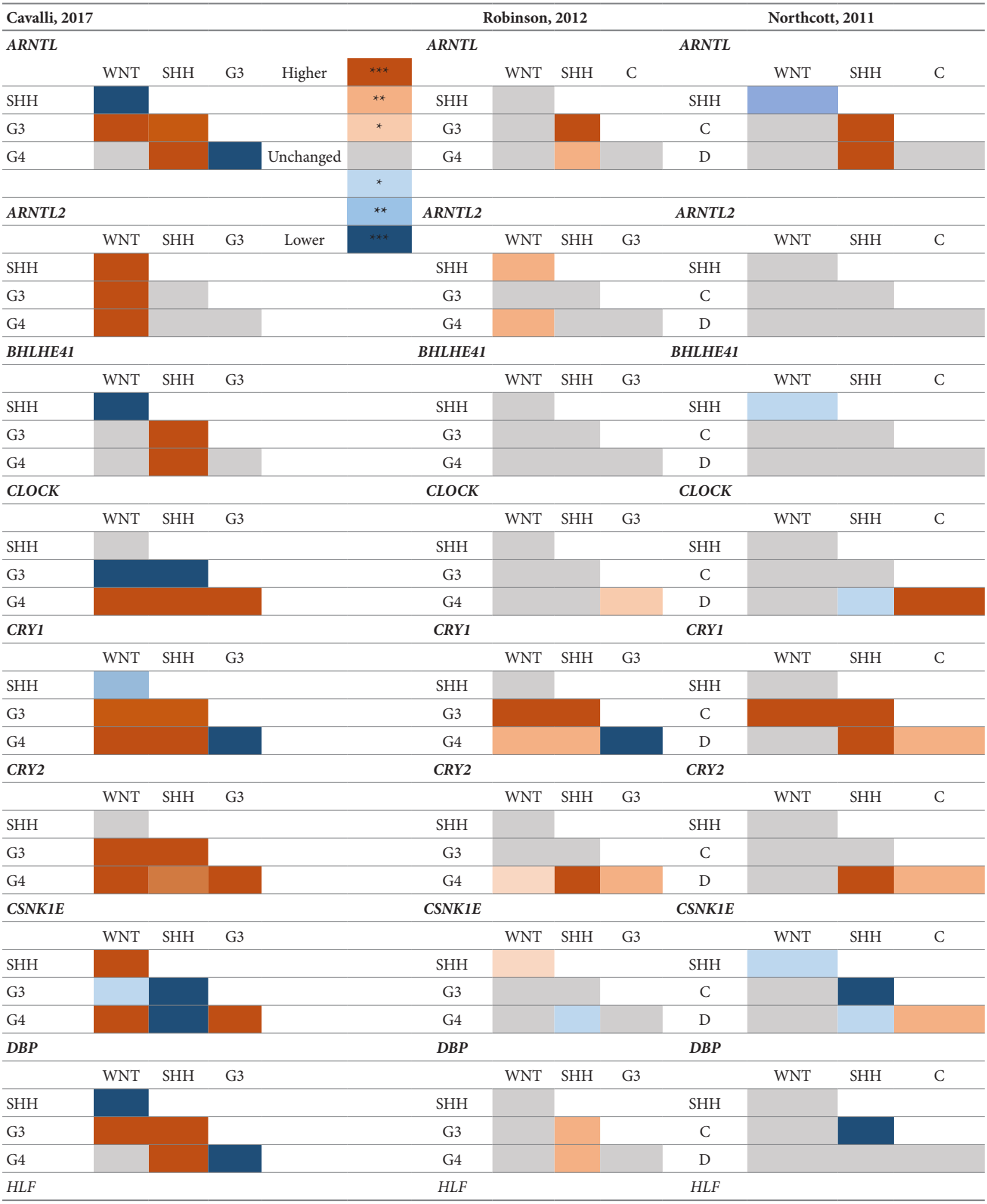


Figure 3. Expression of circadian clock genes in pediatric high-grade glioma (pHGG) cell types. (A) Cluster analysis derived from scRNA-seq data as reported by DeSisto *et al.*[50], showing three major cancer cell types identified as having astrocyte, mesenchymal, or oligodendrocyte progenitor cell (OPC) features along with immune cells show in two large clusters (gray ovals). (B) *RORA* was highly expressed in individual cells of the immune cell clusters, shown as small circles with color intensity indicating increasing expression. Blue circles represent cells lacking detectable expression. (C) The cancer cell clusters also have elevated *RORA* expression. (D) *RORA* transcripts were particularly elevated in immune cell clusters 2 and 22. Labeled cell types are described in the original published study. (E) Average expression of the core clock gene set in the three identified cancer cell types. Cells lacking detectable expression were excluded. (F) Average expression of the core clock gene set in all malignant cells and in all immune (non-malignant) cells. Results and cluster plots were accessed through UCSC Cell Viewer.

through UCSC Cell Viewer (Figure 5A). Like the pairwise comparisons within the bulk MB tumor analyses, the 12 circadian clock genes were more highly expressed overall in the Group 3 and Group 4 subtypes (Figure 5B), which were both significantly higher than the SSH and WNT subtypes (Kruskal–Wallis Test, H = 30.834, $p<0.001$, n = 12, 3 df) as shown in Figure 5C. Like *RORA* and *RORB* in the pHGG scRNA-seq results, the average expression of *RORA* was among the highest of the clock genes in MB cells (Figure 5B).

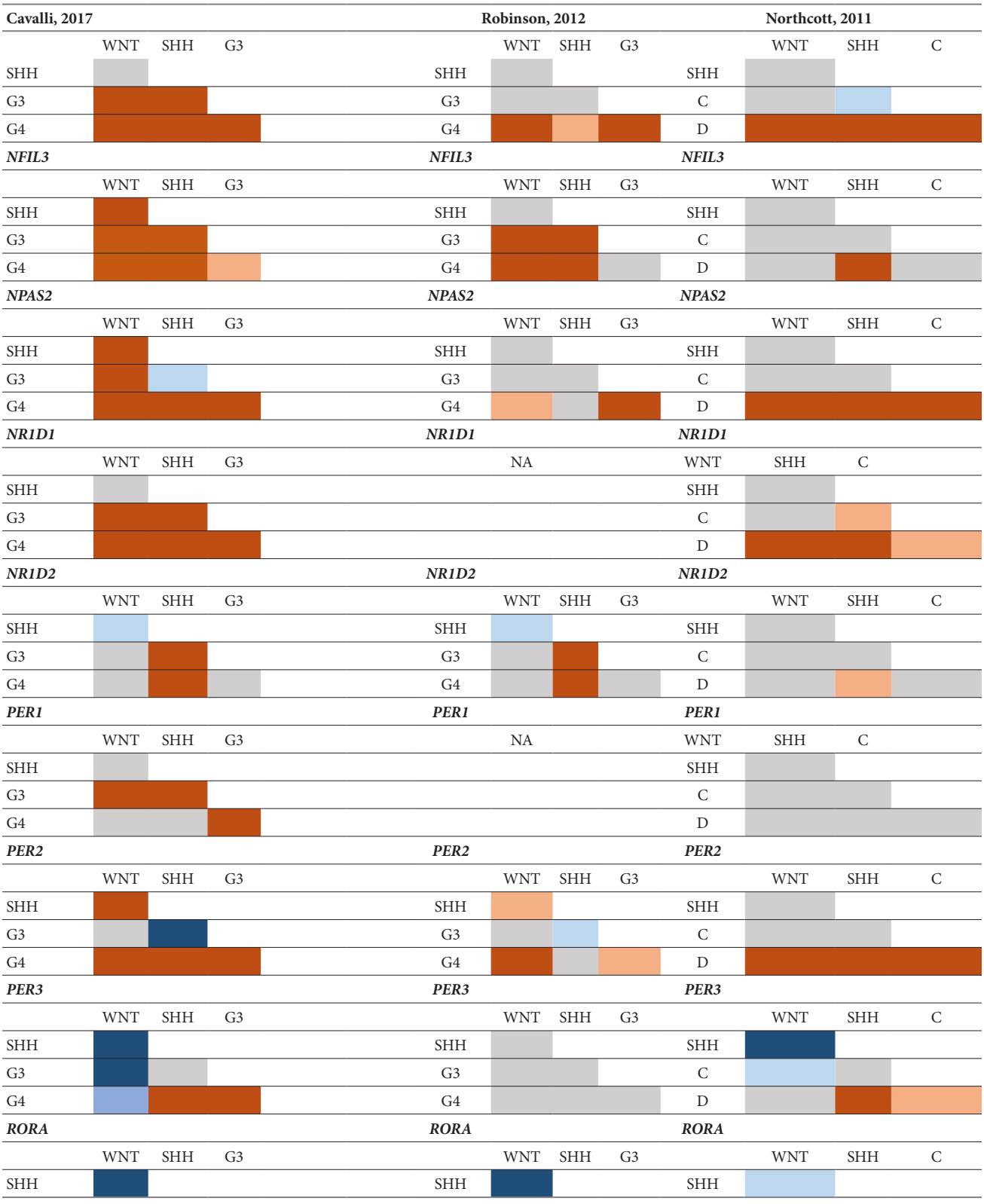
The percentage of cells in the four MB subtypes that had detectable expression of the clock genes varied widely,

Table 4. Pairwise comparisons between medulloblastoma subtypes



(Cont'd...)

Table 4. *(Continued)*



(Cont'd...)

Table 4. ***(Continued)***

Cavalli, 2017				Robinson, 2012				Northcott, 2011			
G3				G3				C			
G4				G4				D			
RORB				***RORB***				***RORB***			
	WNT	SHH	G3		WNT	SHH	G3		WNT	SHH	C
SHH				SHH				SHH			
G3				G3				C			
G4				G4				D			
TEF				***TEF***				***TEF***			
	WNT	SHH	G3		WNT	SHH	G3		WNT	SHH	C
SHH				SHH				SHH			
G3				G3				C			
G4				G4				D			
TIMELESS				***TIMELESS***				***TIMELESS***			
	WNT	SHH	G3		WNT	SHH	G3		WNT	SHH	C
SHH				SHH				SHH			
G3				G3				C			
G4				G4				D			

Notes: Data show here is the significantly higher or lower expression of each member of the circadian gene set, by Tukey's HSD, in the WNT, SHH, Group 3 (G3 or C), and Group 4 (G4 or D) subtypes; *p<0.05; **p<0.01; ***p<0.001; The colors indicate the significance of the difference (higher or lower from the non-cancer controls); Data and statistical tests are from Cavalli *et al.*,[45]; Robinson *et al.*,[46] and Northcott *et al.*[47] in GlioVis.

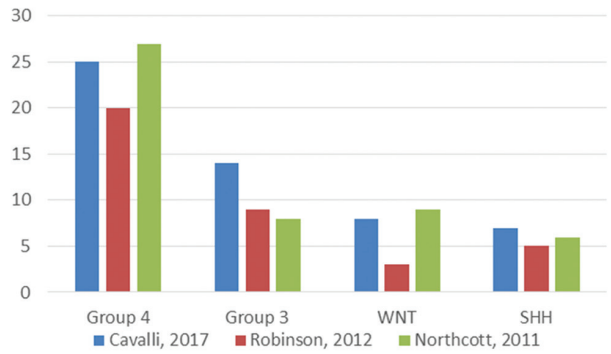


Figure 4. Comparison of the circadian clock gene set expression in four subtypes of medulloblastomas. The bar charts show the number of times a subtype expressed significantly more of one of the 20 genes relative to another subtype. Average counts are as follows: Group 4 = 24 (± 3.60 SD); Group 3 = 10.33 (± 3.21 SD); WNT = 6.67 (± 3.21 SD); and SHH = 6 (± 1 SD). The subtypes have significant differences in their relative expression according to the Kruskal–Wallis test (H = 8.44, p=0.0378). The three replicates for each subtype are from studies by Cavalli *et al.*,[45] Robinson *et al.*,[46] and Northcott *et al.*[47] as shown in GlioVis and Table 4.

from zero to over 30% (Figure 5D). The scRNA-seq results also showed significantly higher expression of the 12 clock genes in non-malignant (immune) cells than in the averaged four MB cell types (1.088 ± 0.2968 vs. 0.7894 ± 0.08655 SD; t-test, p=0.00625, n = 12), with *RORB* and *RORA* having the highest activity. Nevertheless, the percentage of cells in the immune cell clusters expressing the clock genes was significantly lower than in the malignant cells, as shown by averaging across the clock genes and comparing the immune cell average with the average of the four MB subtypes (6.875 ± 6.561 vs. 13.054 ± 7.181 SD; t-test, p=0.0261, n = 12).

We then used the GSE49243 dataset from Kool *et al.*[57] and GEO statistical tools to compare the effect of patient age on clock gene expression in the sonic hedgehog-driven medulloblastoma subtype (SHH-MB). Initially, we compared samples from patients under 18 years of age with those who were 18 or over. None of the differences in expression of the 20 clock genes were significant according to their adjusted p-values. Because the World Health Organization considers adolescence starting in the second decade of life,[58] we then compared clock gene expression in the 21 samples from patients under 10 years of age with the 50 from patients over 10. Several of the clock genes were significantly repressed in the preadolescent (infant and childhood) MB tumors (Table 5).

3.3. Comparing recurrent and original gliomas

Next, we compared the expression of the circadian gene set in recurrent and original tumors. Two ependymoma datasets, from Hoffman *et al.*[51] and Witt *et al.*,[52] and one GBM dataset from Schwartzentruber *et al.*,[53] were analyzed

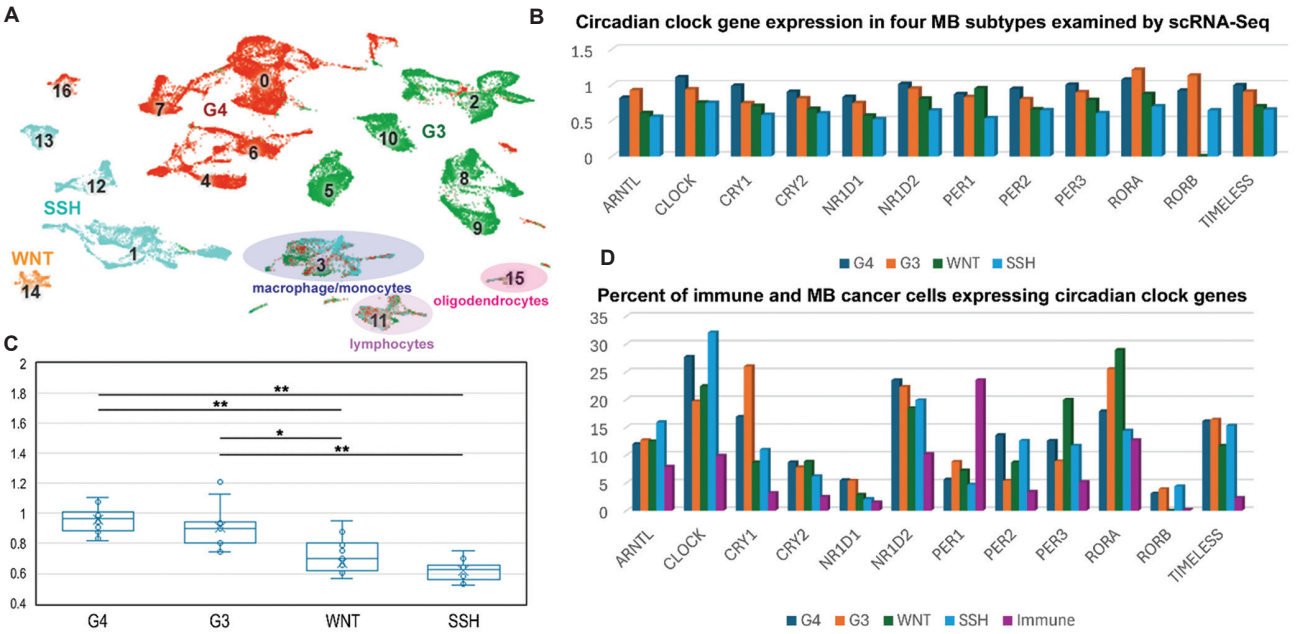


Figure 5. Comparison of the limited clock gene set expression in four medulloblastoma subtypes. (A) Cluster analysis from scRNA-seq data, as reported by Riemondy *et al.*,[49] shows Group 4 (G4), Group 3 (G3), WNT, and SHH subtypes in cells pooled from patient tumor samples and separated from immune cells and other non-malignant cells. (B) Average normalized expression of the core clock gene set in the MB subtypes. Results are from cells with detected gene expression. (C) Clock gene expression is significantly higher in the G3 and G4 subtypes than in WNT and SSH (*p<0.05, **p<0.01). (D) All core clock genes are expressed in the cells identified as immune cells. The percentage of cells expressing the clock genes is lowest in the immune cells, except for *PER1*. Results and cluster plots were accessed through UCSC Cell Viewer.

Table 5. Age-dependent differential circadian clock gene expression in primary SHH-MB

Gene symbol	Adjusted *P* value	Log fold change
CRY2	0.00152	−0.6151
HLF	0.00618	−1.278
NR1D1	0.00277	−0.7031
NR1D2	0.00436	−1.332
PER3	0.00106	−1.258
RORA	0.00151	−0.5289

Note: Patients under 10 years of age versus 10 years and above. From Kool *et al.*[57] and GEO.

in Gliovis. Very few differences were observed when testing with Tukey's HSD, and these were not consistent between ependymoma datasets or when they were compared with the GBM results. The Hoffman dataset showed lower *ARNTL* and elevated *DBP* expression in the recurrent tumors (p<0.05). The Witt dataset showed lower *CRY2* (p<0.01) and lower *DBP*, *HLF*, and *PER3* expression (p<0.05) in the recurrent group. The Schwartzentruber dataset showed higher expression of *ARNTL2* and *PER2* in recurrent tumors (p<0.01). Datasets were also compared in terms of patient gender, and no significant differences were detected in any of these datasets. Finally, the dataset from de Bont *et al.*[54] was also examined, and there were no significant differences between recurrent and original tumors, although in this dataset, GlioVis grouped results from multiple pediatric tumor types in the analysis, thereby possibly masking significant differences. Furthermore, the Witt and Schwartzentruber datasets included some adult patient samples.

4. Discussion

4.1. Consistent clock gene expression patterns observed in adult and pediatric gliomas

More clock genes were significantly repressed than overexpressed in the pediatric and adult gliomas relative to normal tissue, which is consistent with evidence that clock genes are generally downregulated in cancers, perhaps through epigenetic modification.[59-62] Although this reduced expression might impair the functioning of the circadian clock mechanism, GSCs of adult gliomas and cells of several other cancers are rhythmic and appear to depend on their own circadian clock for survival.[9,10,13,14,63-68] Therefore, GSCs in pediatric tumors might remain rhythmic independent of whether the rest of the tumor mass generates a circadian rhythm. When considering the role of circadian genes in any cancer, it is important to consider how they could be acting through the circadian

timing information they generate and disseminate, as well as any cell regulation they provide independent of the clock, particularly in cancer cells that frequently have a compromised circadian clock mechanism. Furthermore, some of the significant differences in gene expression discussed here might depend on the phase of the circadian cycle when the glioma samples were collected, which was not available in this analysis.

Starting with one of the most highly repressed clock genes in the bulk-tumor data of this study, *RORB* appears to be a promising target for additional therapies developed to treat pediatric and adult gliomas. It was significantly underexpressed relative to non-tumor tissue in eight of the ten bulk-tumor tumor studies examined (not Kamoun *et al.*[42] and de Bont *et al.*[54]). Nevertheless, according to the pHGG scRNA-seq dataset, *RORB* was one of the more highly expressed genes when compared with the rest of the core clock gene set. It was one of the least widely expressed genes of the core circadian gene set when comparing cell types in the MB scRNA-seq results. Along with its role in the circadian timing mechanism, RORB may regulate tumor growth through interaction with the Wnt intracellular signaling pathway.[69] However, any treatment designed to increase RORB protein levels or stability might induce *ARNTL*, which would be predicted to favor GSC survival.

CRY2 may be an effective drug target for pediatric gliomas as it was significantly underexpressed relative to non-tumor tissue in 10 of the 11 pediatric bulk-tumor datasets examined. Pharmacological treatments that prevent CRY protein degradation, thereby allowing it to suppress BMAL1/CLOCK functions, have been shown to be effective in suppressing the proliferation of adult GSCs[9] and, we predict, pediatric glioma cells as well.

The *TIMELESS* gene was unique in that it was the most overexpressed gene in pediatric gliomas, indicating it is a promising target for developing treatments that make use of the circadian clock through chronopharmacology or more conventional, non-circadian therapies. Among the medulloblastoma subgroups, *TIMELESS* expression was significantly higher in the G3 and G4 subgroups relative to SHH, suggesting they would be most impacted by treatments suppressing *TIMELESS*. On the other hand, TIMELESS may be at a minimal functioning level in the SHH-expressing cells, indicating greater vulnerability of this subgroup to TIMELESS suppression, if the protein supports cancer cell survival. Results from both the pHGG and MB scRNA-seq studies indicated that a greater understanding of the functioning of TIMELESS activity in gliomas is needed.

Increased *TIMELESS* expression has been reported in gastric cancer,[70] and its elevated expression in adult gliomas is considered a risk factor.[71] *TIMELESS* shows higher expression in high-grade than low-grade gliomas and adjacent non-tumor tissue,[72] in agreement with *TIMELESS* expression we observed in pediatric gliomas. Because it regulates DNA replication and maintains cell migration in other cancers,[73] therapies should be explored that manipulate the TIMELESS protein or its specific binding partners, such as TIMELESS interacting protein (TIPIN). Because TIMELESS is reported to suppress gene induction by the BMAL1/CLOCK dimer,[74] a strategy may be developed like that in which CRY proteins are elevated to interfere with the dimer's induction of genes through E-boxes. Separate TIMELESS-elevating and CRY-elevating compounds might be particularly effective when used in combination to target the same process, which appears critical for cancer cell survival. Altered sleep could be one unintended effect because of reports of *TIMELESS* mutations affecting sleep onset.[75] A recent study indicates a role for TIMELESS in hippocampal learning and memory functions through glutamate receptors,[76] which should be considered when developing a cancer therapy.

4.2. Differences in clock gene expression between adult and pediatric gliomas

4.2.1. ARNTL and GSCs

BMAL1, the product of *ARNTL*, and its dimerization partner CLOCK are together considered critical for circadian rhythm generation and survival of adult GBM cells.[9,29] Therefore, it was unexpected that *ARNTL* would be significantly downregulated in the bulk-tumor pediatric datasets, including pediatric GBM. Nevertheless, BMAL1 is reported to act as a tumor suppressor in an epithelial-type GBM cell line (LN229) used in a mouse xenograft model,[77] which agrees with the significant *ARNTL* suppression we detected. It is possible that BMAL1 suppresses replication of cancer cells in the epithelial state and is then upregulated during EMT, through which mesenchymal GSCs are produced. The lower *ARNTL* expression in the OPC-like cells relative to the mesenchymal cell group of the pHGG scRNA-seq study agrees with this possibility. The reduced overall *ARNTL* expression in the bulk tumor data might be explained by a large percentage of non-stem cells, such as OPCs, in the tumor mass. It has been proposed that tissue-specific conditions and cell-cell interactions may determine whether *ARNTL* and other clock genes act as either tumor suppressors or, instead as oncogenes.[6]

The GSCs generated by EMT may depend on elevated and perhaps rhythmic *ARNTL* expression.[78] In agreement with this speculation, GSC cell cultures have provided

evidence that BMAL1 and other clock proteins enable GBM cell survival.[9,79] In contrast, nasopharyngeal carcinoma cells show greater radioresistance and enhanced post-EMT properties (motility, invasiveness) as BMAL1 levels decline.[80] Regulation by BMAL1 and CLOCK appears to differ between cancer cell types.[79] BMAL1 also serves in the EMT of non-cancerous cells during development. For example, a recent study showed that trophoblasts rely on BMAL1 to undergo EMT, which is needed to anchor the placenta in the uterus, and that CRY2 suppresses this EMT event.[81]

Pharmacological methods that can selectively control BMAL1 or E-box regulation of CCGs are promising treatments for gliomas, particularly to repress EMT and resulting cell motility and invasiveness. However, the CRY protein-stabilizing drug KL001 that suppresses adult glioma cell growth[9] might be less suitable for pediatric gliomas, where *ARNTL* and *ARNTL2* were found to be already repressed; additional interference through elevated CRY proteins and reduced BMAL1 functioning may not be beneficial. However, we did not see evidence of *ARNTL* suppression in the scRNA-seq results, although direct comparisons with expression in normal tissue were not provided.

The difference in *ARNTL* expression between pediatric and adult GBM observed here in the bulk tumor data should be explored further and may indicate that a unique strategy is needed for circadian clock-based treatments that would be specifically designed for pediatric tumors. Notably, *DBP*, *HLF*, and *TEF*, which are induced by BMAL1 and CLOCK, were also significantly repressed in the pediatric and adult gliomas, suggesting that overall CCG regulation by BMAL1 may be impaired. This deficiency might be corrected to improve patient outcomes through a specific pharmacological intervention. *DBP*, *HLF*, and *TEF* were downregulated, and *NFIL3* was upregulated in adult GBM, consistent with the reported antagonistic relationship between NFIL3 and the other three transcription factors.[55] This inverse relationship was not observed in the pediatric gliomas. Although *ARNTL* was suppressed or near normal levels in the MB datasets, it was elevated in Group 3 relative to the SHH and WNT subgroups (Cavalli, Robinson, and Northcott datasets), suggesting differences in how the associated tumor cell types might respond to *ARNTL*-modulating treatments.

The higher expression of clock genes in Groups 3 and 4 in relation to the other medulloblastoma subtypes suggests that they have adequate expression to sustain circadian rhythms intrinsic to these cells, although circadian rhythmicity needs to be demonstrated. Nevertheless, it is conceivable that rhythm generation may be absent despite elevated clock gene expression if certain critical clock genes are constantly activated, thus preventing the necessary troughs in their rhythms and completion of the oscillation. Adequate clock gene expression at least at a minimal level is a necessary but not sufficient criterion for circadian rhythm generation, and overexpression could block rhythmicity.

4.2.2. PER genes and GSCs

In bulk tumor datasets, the pediatric GBM did not show reduced *PER2* and *PER3* expression relative to normal brain tissue, which differs from the adult GBM data examined here, and reported suppression of the *PER* genes in many but not all types of cancers.[6] Although PER protein was not measured, the apparently normal *PER* gene expression in the pediatric gliomas increases the possibility that these cells include functional circadian clocks. Nevertheless, reported rhythms in cell cultures derived from GBM[9,64] indicate that even the suppressed *PER* gene expression in adult GBM is adequate for circadian rhythm generation. The importance of considering *PER2* expression levels in cancer cells depends in part on its ability to bind to and stabilize the tumor suppressor p53, but how it regulates p53 activities is still being actively explored.[82]

These transcriptomic results need to be confirmed through quantitative measures of the expressed proteins and their rhythmicity. However, information on the levels of clock proteins in pediatric proteins is lacking. If pediatric gliomas have more robust and less disrupted circadian rhythms than adult gliomas, then anticancer treatments might prove effective by manipulating the clock mechanism that apparently supports cell survival and helps to maintain the stem cell state. The observed downregulation of *ARNTL* and its paralog *ARNTL2* in pediatric tumors might, however, be impeding the ability of pediatric GBM tumors to generate rhythms.

Along with *PER2*, *PER1* is typically downregulated in gliomas[83] and other tumors, for example, those in breast, prostate, and oral squamous cell cancers.[84-86] *PER1* expression was reported in only one of the five pediatric datasets that were compared with non-tumor tissue. Surprisingly, it was elevated in one of the four adult GBM datasets encompassing this gene, unlike the overall *PER2* and *PER3* downregulation in adult GBMs. The elevated *PER1* expression might be explained by how *PER1* transcriptional regulation differs from that of *PER2* because its promoter responds more effectively to intracellular signals acting through Ca^{2+} and cAMP.[87] This pathway is instrumental in the ability of neurons in the SCN to respond to direct retinal projections through the

optic nerve that entrain the SCN clock to external cycles of light and dark.

PER3 is perhaps the most poorly understood gene of the *PER* family. Of the three *PER* genes, it appears to have the least importance in the circadian clock mechanism, as shown by behavioral and gene knock-out studies in mice.[20] Nevertheless, its suppression in cancers, including gliomas, indicates it could have important functions in processes other than the circadian clock, particularly in cancer cells lacking a functional clock. Although important in tumor functioning, the role of the *PER* genes in cancer remains unclear. For example, *PER3* expression is elevated in U118MG cells derived from an astroblastoma, a pediatric and adult glioma, and *PER3* overexpression and interference showed that migration and invasion abilities are positively correlated with *PER3* expression.[88] Thus, PER3 may play a role in sustaining the stem cell state in tumors.

However, another study reported that overexpression of *PER3* caused a loss of cancer stem cell behaviors and marker proteins, including NOTCH and SOX2, in the HCT-116 colorectal cell line.[89] This result is not, however, contradictory with our findings because the datasets we examined used RNA from bulk tumor tissues and would not be expected to reflect GSC gene expression alone. A particular cell subpopulation that dominates in the whole-tumor RNA examined in these studies would be more highly represented in the results. An analysis of *PER3* specifically in GSCs is needed to resolve the role of this regulator in GSCs. Furthermore, these results do not take into consideration the different states of GSCs, including variations between GSC classes.[90] Furthermore, the different GBM tumor subclasses were not considered, except for the pHGG scSCN-seq study that identified a mesenchymal cell type. The mesenchymal tumor subclass is particularly enriched in GSCs.[91]

4.2.3. Age-dependent expression differences of other clock genes

Considering the other observed differences between pediatric and adult glioma, the lack of elevated CRY1 expression in pediatric GBM and LG bulk tumors suggests a possible path for controlling pediatric tumors that has not been explored. *NR1D2* was often repressed in the GBM datasets but not as extensively in the LG pediatric and adult PA tumors, suggesting that treatment strategies may need to be adjusted according to tumor type. These results were not consistent with reported overexpression in adult GBM cells that also promotes cell proliferation and migration.[92] Nevertheless, Rev-erb-alpha and Rev-erb-beta agonists SR9009 and SR9011, which suppress adult gliomas[93] may also be effective against pediatric GBM and ependymomas, which displayed suppressed *NR1D2*. Expression of the *BHLHE41* (DEC2) gene only showed altered expression (upregulation) in pediatric PA and EP tumors. The DEC2 protein is a transcription factor that interacts with BMAL1 and has been examined in cancer cells along with *BHLHE40* (DEC1).[33,94-96]

In this study, we compared clock gene expression in the pediatric and adult PA tumors. Unlike pediatric PA, *CRY1* and *RORA* were significantly overexpressed in the adult PA. These genes were repressed in one of the pediatric PA datasets, indicating a distinctly altered pattern that may reflect different circadian clock functioning. Some of these differences could be related to the tumor location or tissue of origin. There are reported differences in the preferred locations for PA and EP tumors according to age, with adult PA appearing more often in the cerebrum than cerebellum and adult EP frequently appearing in the spine.[31] Similarly, pediatric and adult medulloblastomas are reported to differ in their expression of the wingless-type (*WNT*) and sonic hedgehog (*SHH*) genes that drive developmental changes and are used to categorize tumor subtypes.[31] The potential treatment of adult GBM mentioned above that suppresses BMAL1 activity by elevating CRY protein levels[9] might also be useful in treating SHH-type MB because we found, using the dataset from Kool *et al.*,[57] that patients under 10 years of age have significantly repressed *CRY2* expression relative to older patients.

The differences in clock gene expression between the pediatric and adult gliomas could result from the very different growth and neurogenesis conditions occurring in these two nervous systems. The transcription factor and stem cell marker SOX2 upregulates circadian clock genes, producing higher amplitude circadian rhythms in *PER* gene expression in the SCN.[97] It may serve the same function in circadian clocks of cancer cells, particularly in GSCs, where elevated SOX2 protein is a known marker. We speculate that SOX2 or related morphogenic genes induced in the developing brain maintains *PER* gene expression in pediatric gliomas.

Recurrent tumors are frequently enriched with stem cell markers,[98] which supports their often more aggressive nature. GSCs *in vitro* display circadian rhythms and elevated expression of some core clock genes such as *ARNTL*.[9] Nevertheless, the pediatric EP and GBM datasets examined here only showed elevated expression of *DBP* in the recurrent tumors, and in only one EP dataset. Although few differences between recurrent and original tumors were observed, this does suggest that any pharmacological tools targeting clock genes may be equally useful for

treating both stages of cancer. Additional studies should examine clock gene expression in recurrent tumors of other glioma types. Furthermore, clock gene expression should be evaluated in recurrent tumors of glioma types not thoroughly examined here, such as DIPG, another high-grade pediatric tumor that appears to be related to GBM.[36]

4.3. Evaluating clock gene activity in pediatric gliomas at different times of day

This study did not attempt to detect changes in circadian rhythms in the various glioma groups relative to non-tumor tissue, which might provide insight into the observed differences in gene expression. What was examined was any significant departures in expression above or below that in equivalent normal tissue. To characterize any circadian rhythms in the pediatric glioma tissue, the tumor cells would, ideally, need to be maintained *in vitro*, perhaps as tumorsphere cultures, and then synchronized through currently available methods so that the cells oscillate together with a common phase of the circadian cycle.[99] As with adult glioma cells, expression would then be assayed in these patient-derived cells at intervals to measure multiple circadian cycles.

A previous study by Huang *et al.*,[100] comparing microarray-assayed transcriptomes of pediatric and adult MB tumors, found by GSEA that the pediatric cells have upregulated expression of a gene set described as "entrainment of circadian clock by photoperiod" (GO:0043153). Several of the members of this probe gene set were also tested in our study (*CRY1*, *CRY2*, *PER1*, *PER2*, and *PER3*). Like their results with MB tumor samples, we found that *PER2* and *PER3* were suppressed in adult GBM relative to normal tissue but not in the two pediatric GBM datasets (Griesing, 2013 and Buczkowicz, 2014). Furthermore, we detected a significant increase of *CRY1* in adult GBM, but not in pediatric GBM, which differs from their MB results. It would be useful to test whether glioma cells use this gene set to entrain their circadian clocks to daily oscillations in hormones, cytokines, nutrients, *etc.*, in their extracellular environment. Evidence supports the entrainment of GBM circadian clocks to daily oscillations in blood cortisol.[11]

It is unlikely that the tumor samples used to produce transcriptomic data were collected from patients at a consistent time of day, suggesting that the values could have fluctuated in response to influences from circadian rhythms in gene expression. The extent of endogenous circadian rhythms in gene expression in pediatric tumor cell types remains unknown. Of course, these conclusions that are drawn mostly from bulk tumor data would benefit from studies capable of spatially resolving gene activity within tumor regions that are known to differ in their stem cell properties.[101,102] It is also unclear whether the circadian clocks in the sampled tumors were disturbed or suppressed by the cancer or, alternatively, the cancer was facilitated by a disrupted clock within the tissue of origin. Much still needs to be explored concerning the circadian clocks functioning within tumors *in situ* and how they interact with the rest of the body, altering rhythms within and beyond the cancer cells.

4.4. Clock gene activity elevated in pediatric glioma subtypes

Examining the relative expression of the clock genes across four recognized MB subtypes provided a detailed view of which subtypes may rely on these genes either in circadian timing or in a non-clock function. In the bulk tumor data, the Group 4 subtype had elevated expression of seven genes relative to the other subtypes, and Group 3 had two. These results were supported by the scRNA-seq analysis that revealed significantly higher expression of circadian clock genes in G3 and G4 than in the SHH and WNT subtypes.

Interestingly, elevated *TIMELESS* expression was also observed more frequently in the G3 and G4 subtypes of the bulk tumor studies, which, along with its higher expression relative to normal tissue in several of the other glioma datasets examined, further argues for its value in developing novel treatment strategies. Nevertheless, the *TIMELESS* expression did not appear to be elevated relative to the rest of the circadian gene set in the pHGG and MB scRNA-seq results. Along with nearly all core clock genes, *TIMELESS* was expressed in a larger percentage of the malignant MB cells than the immune cells, although it is not accurate to consider these stromal cells comparable to normal tissue because of the effects from the tumor microenvironment. An analysis of the spatial expression of TIMELESS protein within the tumor cell types is needed to determine whether its functions are promising targets for anticancer treatments.

Circadian clock genes should be examined for their possible role in any of the critical cancer progression events involving G3 and G4 MB subtypes. G3 cells are considered the most aggressive of the four subtypes, whereas the SHH and WNT subtypes are associated with more favorable patient outcomes.[103,104] The G3 and G4 subtypes appear to be part of a continuum arising during development, as genes expressed in cells derived from specific neural precursors are inappropriately regulated, leading to tumor formation.[105] Also, the G3 and G4 subtypes are more than twice as likely to have metastasized before treatment than are SHH and WNT tumors.[106] Medulloblastoma cells are

particularly dangerous when they metastasize, forming secondary tumors in the meninges and elsewhere.[107]

As described in an extensive review of GSCs in medulloblastomas,[108] GSCs are present in all four medulloblastoma subtypes, and distinguishable GSC categories are present. For example, cells expressing either of the GSC markers CD133 and CD114 appear as distinct cell populations. Anticancer treatments acting through epigenetic modifications of genes that maintain stemness are being tested, particularly histone deacetylase inhibitors.[109] Organoid cultures have been developed to address the role of GSCs in medulloblastoma and other pediatric gliomas.

4.5. Limitations of the current study and future directions for exploration

The present study preliminarily explored circadian clock gene expression in some of the most common pediatric gliomas, embarking on a journey to address the knowledge gap. It is important to continue this approach by examining additional samples from DIPG, currently known as diffuse midline glioma (DMG), which is a pHGG that is histologically similar to GBM,[110] highly lethal, and has poor treatment options. DIPG is often medically treated like GBM, resulting in poor outcomes, but is distinctly different and is reported to consist of three subgroups.[36] Our preliminary results suggest that some of the clinically relevant differences between pediatric GBM and DIPG may be their differential expression of circadian clock genes.

Some medications that target glioma subtypes are being tested, such as against the SSH MB subtype.[57] It may be useful to further target subtypes by exploiting their differences in clock gene expression and potential for circadian rhythmicity, as suggested by the current study. Most importantly, because of the daily oscillations in activity of members of the circadian gene set, they should be evaluated in respect to the time of day when samples were collected and, perhaps, at some future point, the phase of circadian clocks assayed *in situ* within patient tumors. More detailed proteomics of the oscillating clock components within tumors is also needed because of the many post-transcriptional effects that can impact circadian rhythms.[111]

5. Conclusion

Pediatric and adult gliomas show some similarity in their expression of core and related circadian clock genes. *CRY2* and *RORB* are the two core clock genes that are suppressed in all three glioma categories: adult GBM, pediatric GBM, and LG glioma. Consequently, pharmaceuticals that are currently being tested as acting through core circadian clock genes could also prove effective in treating pediatric GBM and LG tumors. The lack of *PER2* and *PER3* repression in pediatric GBM and LG tumors, along with other differences, suggests that an alternative drug development approach specific for pediatric gliomas may be needed. The overexpression of *TIMELESS* in all three glioma categories indicates another possible path for effective drug development. Finally, the higher clock gene expression in MB tumors detected in the Group 3 and Group 4 subtypes, and with adolescent onset, may provide opportunities for specialized treatments.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Investigation: Austin Tyler Vogt, Veda Sanjay Mohite, Michael Eric Geusz
Methodology: All authors
Writing – original draft: Austin Tyler Vogt, Veda Sanjay Mohite, Michael Eric Geusz
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable. All datasets were derived from data repositories available to the public.

Availability of data

Original data used in this study can be obtained from the GlioVis data portal for visualization and analysis of brain tumor expression datasets (http://gliovis.bioinfo.cnio.es/), the TIMER2.0 website, (http://timer.comp-genomics.org/timer/), GEO (https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html), and the University of California, Santa Cruz Cell Explorer website (https://www.pneurooncellatlas.org/). The collected and analyzed data are available from

the authors upon request.

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ORIGINAL RESEARCH ARTICLE

Molecular binding of 11q to NS2B–NS3 proteases of dengue and West Nile viruses

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Abstract

Dengue virus (DENV) and West Nile virus (WNV) are mosquito-borne pathogens that cause severe health burdens globally. Despite their impact, no clinically approved antiviral therapies are currently available. The NS2B–NS3 protease is essential for viral genome replication in both viruses, increasing viral loads in infected individuals. Therefore, targeting and inhibiting this protease would significantly reduce viral replication. In a recent molecular dynamics (MD) simulation study, N-(((2,6-dibromophenyl) amino) methyl)-4-morpholinobenzamide (11q) was found to bind more strongly to the NS2B–NS3 protease of the Zika virus (ZIKV) than SYC–1307, a known ZIKV protease inhibitor. Notably, 11q was also observed to inhibit influenza virus replication. Given the high structural and sequence similarity of the NS2B–NS3 protease across ZIKV, DENV, and WNV, it was necessary to evaluate whether 11q can bind to the proteases of DENV and WNV to inhibit their activities. Using molecular docking, MD, and binding free energy studies, we found that 11q strongly binds to the NS2B–NS3 proteases of DENV and WNV with binding free energies of −15.80 ± 3.34 kcal/mol and −13.13 ± 2.56 kcal/mol, respectively. The slightly more favorable binding of 11q to the DENV protease is comparable to that observed with the ZIKV protease. Interestingly, the binding affinities of 11q for all three viral proteases surpass that of the ZIKV–SYC–1307 complex. Therefore, it is proposed that 11q may act as a pan-antiviral agent against ZIKV, DENV, and WNV proteases. However, experimental verification of its protease inhibition activities is required before it can be repurposed for therapeutic use against these viral diseases.

Keywords: Dengue virus; West Nile virus; NS2B–NS3 protease; Docking; Molecular dynamics simulation; Multiple targeting inhibitor

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1. Introduction

Dengue virus (DENV) is a member of the Flaviviridae family and is mainly transmitted through mosquito bites, particularly by *Aedes albopictus* and *Aedes aegypti*.[1] DENV has four distinct serotypes (DENV 1 – 4)[2] that are responsible for inducing viral diseases,[3-5] primarily in tropical and subtropical territories.[4] DENV infections can induce mild to severe effects.[5-7] Similarly, the West Nile virus (WNV) is another clinically significant member of the Flaviviridae family that shares several virological and epidemiological features with DENV but exhibits distinct characteristics. WNV is

primarily transmitted through mosquito bites, but WNV infection can also be contracted through alternative routes, such as blood transfusion, organ transplantation, and vertical transmission. Although most WNV infections are asymptomatic, a small proportion (~1%) can lead to severe neuroinvasive diseases, such as encephalitis, meningitis, or acute flaccid paralysis.[6] Therefore, there is a critical need to design and develop effective antiviral compounds to treat these infections. However, no approved medications are currently available for these diseases.

It is known that the NS2B–NS3 protease of the DENV plays a prominent role in disease progression by cleaving the viral polyprotein chain to generate virulent proteins,[7-9] thereby facilitating viral genome replication in host cells.[10,11] The crystal structure of the DENV–NS2B–NS3 protease (Protein Data Bank [PDB] ID 3U1I)[12] provides detailed structural insights that can aid in the design of antiviral compounds to inhibit protease activity. The DENV protease contains two subunits (chains A and B),[7] which are NS2B (chain A) and NS3 (chain B). NS3 is a large protease domain (182 residues), bound to its cofactor NS2B (45 residues) that wraps around NS3. The NS2B cofactor inserts hydrophobic patches into the protease core to stabilize its active conformation.[13,14] NS3 adopts a chymotrypsin-like fold, composed of two β-barrel domains, and contains a catalytic triad – H51, D75, and S135 – essential for enzymatic cleavage of the viral polyprotein. In addition to its catalytic role, NS3 contributes significantly to substrate recognition and binding. Residues, such as D129, F130, A132, G151, G153, and Y161, are involved in substrate binding. Similarly, several residues from the NS2B cofactor, such as D81, G82, and T83, contribute to stabilizing substrate binding.[7-9,15]

The NS2B–NS3 protease of the WNV plays a similar role in disease progression. It also contains NS2B (chain A) (47 residues) and NS3 (B chain) (186 residues) (PDB ID 2FP7).[13] Remarkably, both the DENV and WNV proteases share significant similarities at the genomic, structural, and functional levels.[3,16-18] For example, both viruses possess a positive-sense single-stranded RNA genome of approximately 11 kilobases, which encodes a single polyprotein. This polyprotein is post-translationally cleaved into three structural proteins – envelope, precursor membrane, and capsid – and seven non-structural proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.[19-27] Structural alignments of NS2B–NS3 proteases of DENV and WNV (Figure 1) suggest that their catalytic residues (S135, H51, and D75) are conserved. These residues are also conserved for the Zika virus (ZIKV) protease (Figure 1). Other than these catalytic residues, D130, T134, and S136 are also conserved in ZIKV, DENV, and WNV proteases (Figure 1).

Despite several efforts, there are currently no clinically approved small-molecule inhibitors that effectively target the NS2B–NS3 protease of DENV and WNV. Although several promising compounds, such as BP2109,[28] glycyrrhizin acid derivatives,[29] 8-hydroxyquinoline derivatives,[30] and myricetin,[31] have demonstrated inhibitory activities *in vitro*, these compounds possess severe limitations, including a lack of specificity, low potency, poor pharmacokinetic properties, and a failure to target the dynamic active site of the protease.[32] Therefore, there is a need to identify novel small molecules that can tightly bind to the active site of the protease to inhibit its activity.

In a recent study, several derivatives of aryl benzoyl hydrazide were synthesized to target different influenza viruses.[33] Among these derivatives, 10b, 10c, 10g, 11p, and 11q showed encouraging activities against the avian H5N1 flu strain. Among them, N-(((2,6-dibromophenyl) amino) methyl)-4-morpholinobenzamide (11q) (Figure 2A) showed nanomolar antiviral activities against H1N1 and flu B viruses.[33] It also showed excellent bioavailability against the influenza A virus.[33] Remarkably, the binding of 11q to the ZIKV NS2B–NS3 protease was found to be about 5 kcal/mol more stable than that of SYC-1307, a known protease inhibitor[34] (Table 1). Since SYC–1307 inhibits the ZIKV protease with a half-maximal inhibitory concentration value of 0.2 ± 0.01 μM,[35] the stronger binding affinity of 11q suggests that it may inhibit the ZIKV protease more efficiently than SYC–1307. Interestingly, due to the structural similarities between ZIKV, DENV, and WNV, SYC–1307 was also reported to inhibit the proteases of DENV (serotype-3) and WNV, with half-maximal inhibitory concentration values of 0.52 ± 0.06 and 0.78 ± 0.02 μM, respectively.[35]

Given that ZIKV, DENV, and WNV proteases share identical sequences (Figure 1) and structures,[36,37] it was necessary to examine whether 11q can bind to the NS2B–NS3 proteases of DENV and WNV with similar affinity as observed for ZIKV protease. We have recently shown that paritaprevir binds tightly to the proteases of ZIKV, DENV, and WNV, with binding free energies of −11.51 ± 2.82 – −17.3 ± 2.55 kcal/mol,[22] suggesting its potential as a pan-antiviral agent against ZIKV, DENV, and WNV infections. Hence, it was necessary to evaluate whether 11q could act as a pan-antiviral agent against these infections.

2. Materials and methods

2.1. System preparation

The X-ray crystal structures of the NS2B–NS3 protease of the DENV (PDB ID 3U1I)[12] and the WNV (PDB ID 2FP7)[13] were retrieved from the PDB (www.rcsb.org/)

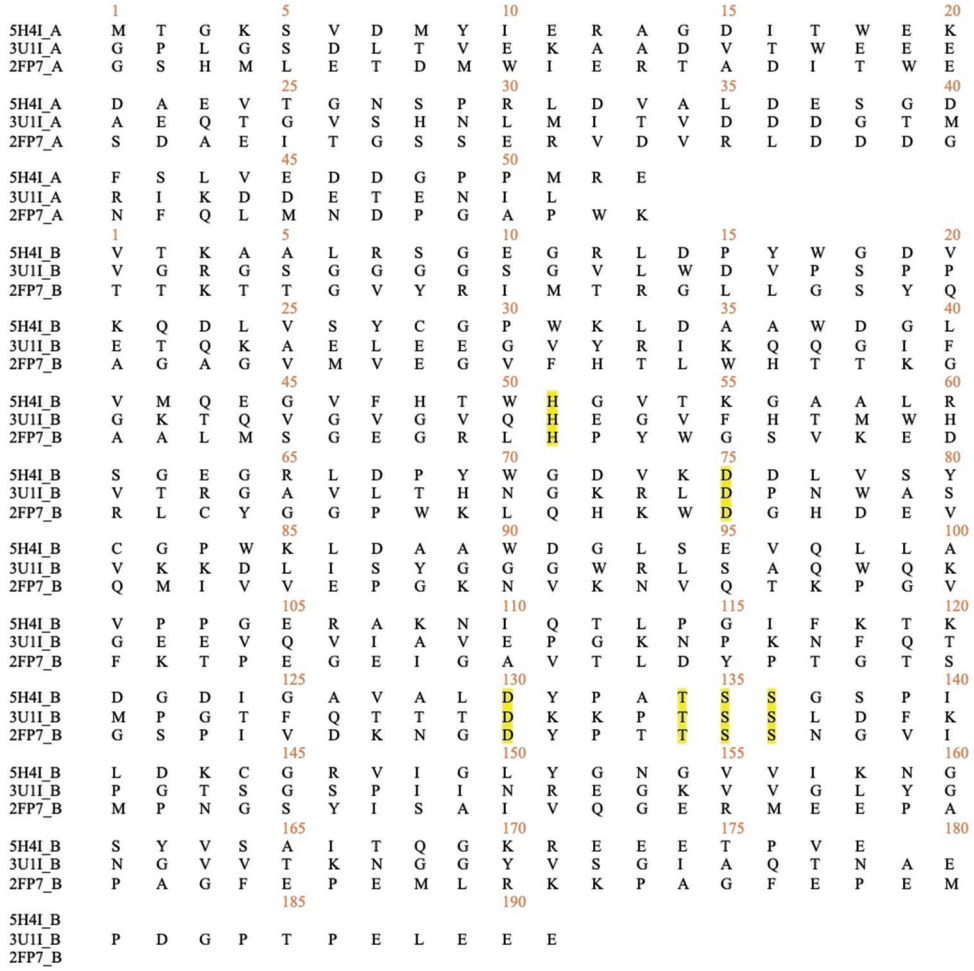

Figure 1. Sequence alignment of dengue virus (Protein Data Bank [PDB] ID 3U1I), West Nile virus (PDB ID 2FP7), and Zika virus (PDB ID 5H4I) proteases. Conserved residues are highlighted in yellow color. A and B chains stand for NS2B and NS3, respectively.

to serve as the initial templates for molecular modeling. The co-crystallized ligands and water molecules were removed from the protein to generate the isolated protease structures. To address any missing terminal residue and improve the structural stability, the N-terminal was capped with an acetyl group, and the C-terminal was capped with an N-methylamide group. Hydrogen atoms were subsequently added to the protease using AutoDock Vina (version 1.5.7).[38] The three-dimensional structure of 11q was geometrically optimized using density functional theory at the B3LYP/6-31G** level in an aqueous medium.[34] The integral equation formalism of the polarized continuum model[39] was used to model the aqueous medium. The Gauss View 5.0 program[40] was used for the structure visualisation of 11q. To generate the DENV–11q protease and WNV–11q protease complexes, the average simulated structure of the ZIKV–11q complex[34] was superimposed onto the isolated protease crystal structures of DENV (PDB ID 3U1I)[12] and WNV (PDB ID 2FP7) to save the coordinates of 11q bound to the proteases of DENV and WNV.[13] 11q was also docked into the active sites of DENV and WNV proteases to ensure that both processes (superimposition and docking) generated the same complex.

2.2. Molecular docking

A grid box of size 15 Å × 15 Å × 15 Å with a grid spacing of 0.375 Å was generated using the AutoDock Tools graphical user interface program.[38] The coordinates of the grid center along the X-, Y-, and Z-axes were set to −4.183, 4.685, and −17.043, respectively, to encompass the known active site of the protease. This grid box was then used to create a score grid based on the ligand structure, enabling a significant reduction in computational time during the simulation, where the configuration file included grid box attributes, along with protein and ligand information. Notably, the AutoDock Vina[38] uses an iterated local search global optimizer for docking,[41,42] treating ligands as flexible while maintaining the protein rigid to accurately predict

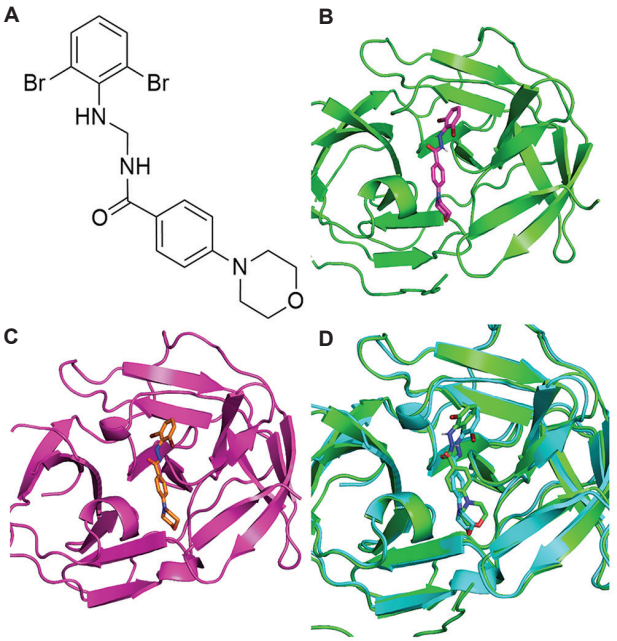

Figure 2. Structure and docking poses of 11q. (A) The two-dimensional structure of 11q. (B) The docked conformation of the dengue virus NS2B–NS3–11q complex. (C) The docked conformation of the West Nile virus NS2B–NS3–11q complex. (D) Superimpositions of docked conformations of NS2B–NS3–11q complexes belonging to dengue and West Nile viruses.

Table 1. Docking scores and binding free energies of 11q bound to the NS2B–NS3 proteases

Complexes	Docking score (kcal/mole)	Binding free energies (kcal/mol)
DENV–NS2B–NS3–11q	−6.5	−15.80±3.34
WNV–NS2B–NS3–11q	−6.2	−13.13±2.56
ZIKV–NS2B–NS3–11q[34]	−6.4	−15.59±3.53
ZIKV–NS2B–NS3-SYC-1307[34]		−11.26±2.84
WNV protease-ritonavir[22]		−7.43±2.16
WNV protease-paritaprevir[22]		−17.3±2.55
DENV protease-ritonavir[22]		−11.51±2.82
DENV protease-paritaprevir[22]		−12.76±2.91

Abbreviations: DENV: Dengue virus; WNV: West Nile virus; ZIKV: Zika virus.

binding conformations.

Initially, the docking protocol was validated by docking the co-crystalized ligand N-benzoyl-norleucyl-lysyl-arginyl-N-[amino(imino) methyl]-N-[(2S)-5-carbamimidoyl-1-hydroxypentan-2-yl]-L-ornithinamide against the DENV protease to reproduce the experimental complex structure (PDB ID 3U1I).[12] As the docking results reproduced the experimental complex structure,[12] the docking protocols were used to dock 11q against the DENV protease to generate the NS2B–NS3–11q complex (Figure 2B). The same protocol was used to dock 11q against the WNV protease to generate the 11q–NS2B–NS3 protease complex (Figure 2C). Superimpositions of both DENV–11q and WNV–11q protease complexes (Figure 2D) reveal that 11q would bind to both the proteases in a similar manner. Interestingly, the docked conformation of 11q bound to proteases of DENV and WNV was similar to the corresponding complexes generated by the superimposition method.

2.3. Molecular dynamics (MD) simulations

Since the protease was considered rigid and solvation effects were not applied during docking, it was essential to assess the influence of protein dynamics and solvent interactions on the binding of 11q with the NS2B–NS3 proteases of the DENV and WNV using MD simulations. The MD simulations were conducted in several steps. First, the 11q–NS2B–NS3 protease complexes were solvated in a cubic water box of size 10Å, and the TIP3P model[43,44] was used to model water molecules. Sufficient counter ions (Na^+ and Cl^-) were added to neutralize solvated complexes. The Assisted Model Building with Energy Refinement (AMBER) force field (ff14SB)[45] of the AMBER 14 program[45] was used to model the protein. To generate force fields for 11q, the General AMBER Force Field method[46] and the Austin Model 1 with Bond Charge Corrections charge model[47] were used. Subsequently, the steepest descent methods were employed to minimize the solvated complexes in 500 steps.[48] The conjugate gradient method was applied for 1,000 steps to further minimize the above-solvated complexes.[49] Initially, only the water molecules were minimized by restraining the protease–ligand complexes using a force constant of 50 kcal/mol/Å^2. Next, the ligand and water molecules were minimized by restraining the protease with a force constant of 50 kcal/mol/Å^2. Subsequently, all molecules were minimized by removing restraints. All complexes were slowly heated to achieve the room temperature of 300 K. During heating, the protease and 11q were first restrained using a force constant of 20 kcal/mol/Å^2 in the number of particles–volume–temperature ensemble for 20 ps. In the second step, the system was equilibrated in the number of particles–pressure–temperature ensemble by applying a harmonic restraint of 5 kcal/mol/Å^2 while keeping the temperature constant at 300 K for 100 ps. During simulations, temperature was controlled using the weak-coupling method,[50] and a constant pressure of 1 atm was maintained using the Barendsen barostat.[50] In the third step, the system was equilibrated for 1 ns by removing restraints. Finally, all complexes were subjected to production runs up to 100 ns using the number of particles–pressure–temperature ensemble and keeping the

temperature at 300 K and the pressure at 1 atm. During each step, hydrogen atoms were restrained by applying the SHAKE algorithm.[51] An integration time step of 2 fs was considered throughout the simulations. To account for the long-range electrostatic interactions, the particle–mesh Ewald approach[52] was employed, and a threshold of 10 Å was considered to account for the non-bonded intermolecular interactions.

2.4. Binding free energy calculations

The Poisson–Boltzmann surface area continuum solvation (PBSA) method combined with the molecular mechanics energy (MM)[53] of the AMBER 14 program[45] was used to compute the Gibbs ΔG_{bind}. The last 10 ns of the MD trajectory were used to extract 100 snapshots of each complex at a pause of 100 ps to compute ΔG_{bind}. For this purpose, the water molecules and ions were stripped from the MD trajectories. Equation I was used to compute ΔG_{bind} as follows:

$$\Delta G_{bind} = G_{cocmplex\ (minimized)} - G_{protein\ (unbound,minimized)} - G_{ligand\ (unbound,minimized)} \quad (I)$$

In this case, ΔG_{bind} represents the binding free energy, $G_{complex(minimized)}$ represents the MM/PBSA free energy of the minimized complex, $G_{protein\ (unbound,\ minimized)}$ represents the MM/PBSA free energy of the minimized protein following its release from its bound ligand, and $G_{ligand\ (unbound,\ minimized)}$ represents the MM/PBSA free energy of the minimized ligand following its release from the complex. Since normal mode analysis was not considered, entropy contributions were absent from the ΔG_{bind}.

3. Results and discussion

3.1. Root mean square deviation (RMSD)

The RMSD of the protein C_α atoms of different residues of the DENV and WNV proteases (Figure 3A), computed by considering the corresponding minimized complexes as references, suggests that the protein does not move much during the MD simulations from its initial conformation. RMSD did not cross 2.5Å, manifesting the protein stability upon ligand binding. If we compare structural variations of the protein C_α atoms in the WNV and DENV proteases, it is clear that the WNV protease has slightly higher RMSD variations. This is because the WNV protease adjusts its conformation to accommodate 11q in its active site and hence is more flexible compared to the DENV protease.

3.2. Root mean square fluctuation (RMSF)

The RMSF of the protease residues (Figure 3B) suggest that the terminal residues are more flexible than the internal residues and therefore have higher RMSF values. Internal residues connected to disordered amino acids and residues belonging to loop regions also possess higher RMSF values. As these residues are far from the active site, they have minimal effects on the complex stability. Nevertheless, none of the terminal residues have RMSF values >20Å, and therefore, do not overreact to the solvent environment.

Overall, the residues of WNV protease demonstrated higher fluctuations with peaks reaching up to ~18 Å, while the residues of DENV protease exhibited lower residue fluctuations across the trajectory, generally remaining below ~14 Å, with several regions showing noticeably reduced flexibility compared to WNV–NS2B–NS3–11q. This, along with RMSD values, suggests that DENV protease adopts a more rigid complex conformation, likely contributing to enhanced binding affinity of 11q.

3.3. Radius of gyration

The radius of gyration (Rg) plots (Figure 4) provide insights into the compactness and overall structural stability of the protein-ligand complexes during the 100 ns MD simulation. As shown in Figure 4A, the DENV–NS2B–NS3–11q complex maintained Rg values ranging between ~16.5 Å and 17.5 Å throughout the simulation. Similarly, the WNV–NS2B–NS3–11q complex exhibited Rg values lying between ~15.5 Å and 16.5 Å (Figure 4B). These results indicate that during the simulations, both the proteases adopted a compact structure, and the compactness of the WNV protease is slightly higher than that of the DENV protease. It also indicates that after ligand binding, the folding structure of the proteases remained intact during the simulations.

3.4. Solvent accessible surface area (SASA)

The SASA plots depicted in Figure 4 reveal that the proteases were well exposed to the solvent throughout the simulations. The SASA values of the DENV protease were computed to be between ~8,500 Å^2 and 10,000 Å^2, suggesting a dynamic but moderate surface exposure during the simulation (Figure 4A). These variations may correlate with subtle local conformational rearrangements, possibly to optimize ligand binding. In contrast, the WNV–NS2B–NS3 protease showed relatively constant SASA values (~9,000 Å^2) with minimal fluctuations (Figure 4B).

3.5. The binding of 11q with DENV protease

The average MD-simulated structure of the NS2B–NS3–11q complex belonging to DENV (Figure 5A) reveals that the ligand 11q remained intact in the active site of the protease throughout the simulations. Notably, the head benzyl group of 11q remained consistently anchored in

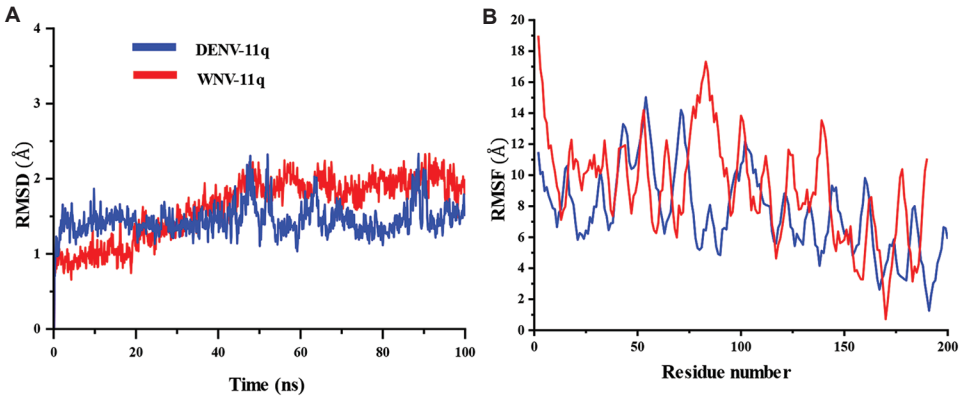

Figure 3. Variations of root mean square deviations and root mean square fluctuations with simulation time. (A) The root mean square deviations (Å) of the protease C_α atoms and (B) the root mean square fluctuations (Å) of the protease residues in the NS2B–NS3–11q protease complexes involving dengue virus and West Nile virus, computed using the minimized complex structures as references.

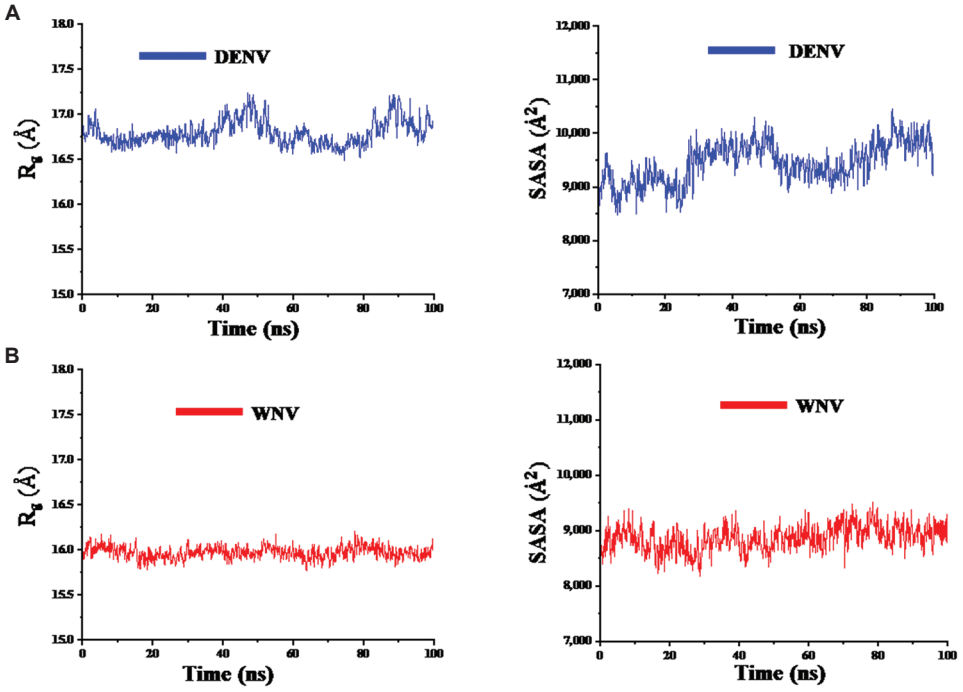

Figure 4. Variations of radius of gyration and solvent accessible surface area with simulation time. (A) Radius of gyration (Rg, Å) and solvent accessible surface area (SASA, Å²) of the dengue virus protease bound to 11q. (B) Rg and SASA of the West Nile virus protease bound to 11q.

the S1 subsite, while the tail ring rotated from the S2 site toward the S1 subsite, indicating an adaptive binding mode as it transitions from the docked pose to the dynamically equilibrated structure observed in MD simulation (Figure 5A). This reorientation also suggests that 11q may optimize its interactions with the surrounding residues over time, enhancing its binding stability. In the MD confirmation, 11q formed two weak hydrogen bonds with residues G133 (43% occupancy) and S135 (22% occupancy), which contributed to the stabilization of the ligand within the binding pocket. Although the hydrogen bond with G133 is slightly weak, it is reasonably steady and can play a critical role in stabilizing the ligand-protein complex. In addition, 11q formed strong π–π stacking interactions with Y161 and H51. These interactions likely reinforced the ligand's positioning and contributed significantly to its overall binding affinity. As a result, a favorable binding free energy of −15.8 ± 3.34 kcal/mol was obtained for the NS2B–NS3–11q protease (Table 1). This energetic profile supports the potential of 11q as a

potent inhibitor of the DENV–NS2B–NS3 protease, with implications for antiviral drug development. Per-residue decomposition of free energy suggests that the P132, followed by S135 and Y161, significantly contributed to the stability of 11q (Figure 5B).

3.6. The binding of 11q with WNV protease

Interestingly, the MD simulations revealed that 11q retained a conformation that closely resembled its initial docked pose throughout the simulation timeframe (Figure 6A). The head benzoyl group remained firmly engaged with the S1 subsite, while the tail aromatic ring continued to interact with the S2 subsite of the WNV–NS2B–NS3 protease active site. This suggests a stable and persistent binding orientation of the ligand during the dynamics process. The molecular interactions stabilizing 11q within the WNV protease included weak hydrogen bonding interactions with T132 (36% occupancy) and S135 (16% occupancy), which, although not dominant, are crucial in maintaining the ligand's orientation in the binding pocket. Furthermore, the π-π stacking interactions with Y161 significantly contributed to the overall binding affinity, reinforcing the ligand's position in the active site. As a result of these cumulative interactions, the calculated binding free energy of the NS2B–NS3–11q complex of WNV was found to be −13.13 ± 2.56 kcal/mol

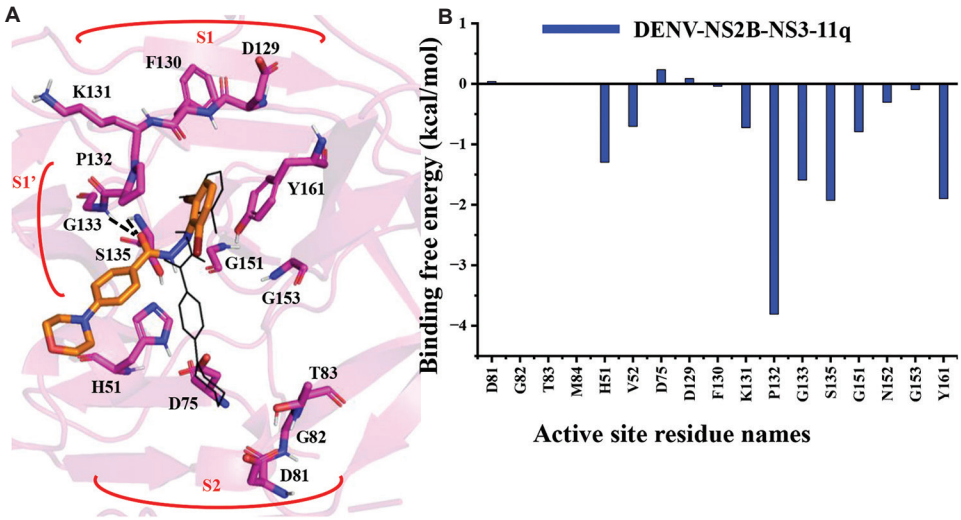

Figure 5. Binding pattern of 11q with dengue virus (DENV) protease. (A) Interactions of 11q with the active site residues of DENV–NS2B–NS3 protease. (B) Per-residue decomposition of binding free energy.

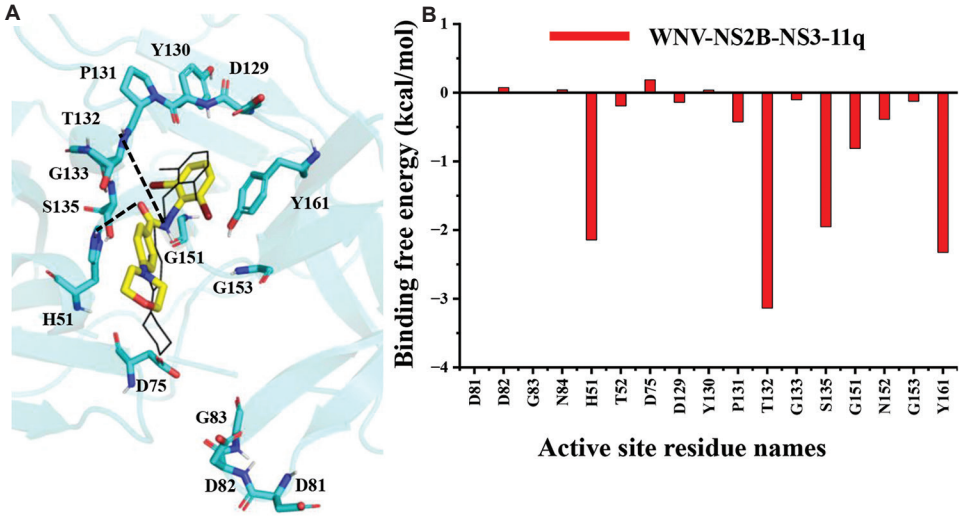

Figure 6. Binding pattern of 11q with the West Nile virus (WNV) protease. (A) Interactions of 11q with the active site residues of WNV–NS2B–NS3 protease. (B) Per-residue decomposition of binding free energy.

(Table 1), indicating a strong and energetically favorable binding affinity. Per residue decomposition of binding free energy suggests that T132, followed by Y161, substantially contributes to the binding free energy (Figure 6B).

If we compare the docking scores and binding free energies of different complexes studied herein (Table 1), it is evident that 11q binds more favorably to the DENV protease than the WNV protease. The binding affinity of DENV–11q protease is similar to that of ZIKV–11q protease complex (Table 1). This implies that 11q would bind to DENV protease as strongly as the ZIKV protease, which in turn will be more stable than the known protease inhibitor SYC–1307.[34] In an earlier study,[22] an antiviral drug, ritonavir, was shown to bind to the proteases of DENV and WNV with binding free energies of −11.51 ± 2.82 kcal/mol and −7.43 ± 2.16 kcal/mol, respectively. Similarly, another antiviral drug, paritaprevir, was shown to bind to DENV and WNV proteases with binding free energies of −12.76 ± 2.91 kcal/mol and −17.3 ± 2.55 kcal/mol, respectively.[22] These results indicate that 11q would form complexes with DENV protease that are about 4 kcal/mol more stable than those formed with ritonavir, and about 3 kcal/mol more stable than with paritaprevir (Table 1). Similarly, 11q binds to the WNV protease with a binding free energy approximately 6 kcal/mol more favorable than ritonavir, but about 4 kcal/mol less favorable than paritaprevir (Table 1). Therefore, 11q would serve as a better inhibitor of DENV protease than ritonavir and paritaprevir.[22] However, although 11q would be a more efficient inhibitor of WNV protease than ritonavir, it would be less effective than paritaprevir.[22]

In an earlier study, the drug-likeness of 11q was assessed by calculating all parameters defined by Lipinski's Rule of 5.[34] It was found that the computed molecular weight, lipophilicity (measured by logP), and the number of hydrogen bond donors and acceptors of 11q did not violate Lipinski's Rule of 5.[34] Similarly, the pharmacokinetic properties of 11q were computed using absorption, distribution, metabolism, and excretion properties. Notably, the gastrointestinal absorption, blood–brain barrier permeability, potential interactions with key cytochrome P450 enzymes, and activity of efflux transporters are factors in predicting drug metabolism and safety. It was found that 11q exhibits favorable gastrointestinal absorption, limited blood–brain barrier penetration, no central nervous system-related side effects, and no significant inhibitory interaction with major cytochrome P450 isoforms.[34] The combined absorption, distribution, metabolism, excretion, and toxicity profile indicates that 11q may act as a promising and safe therapeutic candidate against DENV and WNV infections. These results are in agreement with earlier biochemical studies, where 11q was found to be safe for humans.[33]

4. Conclusion

The NS2B–NS3 proteases of DENV and WNV are important targets for antiviral drug discovery. The present study highlights that 11q can bind to the substrate active site of the NS2B–NS3 proteases of the DENV and WNV firmly, with binding free energies ranging between ~−13.13 ± 2.56 kcal/mol and −15.80 ± 3.34 kcal/mol. The binding of 11q was found to be mainly stabilized by stacking interactions involving Y161 and H51 and hydrogen bonding interactions involving S135, G133, and T132. Notably, despite identical structures and sequences of the active site of the DENV and WNV proteases, their protein dynamics were distinct. Because of this, 11q adopted different conformations in the DENV and WNV protease active sites. Remarkably, the binding affinity of 11q was found to be identical for ZIKV and DENV proteases, which is significantly higher than that of SYC–1307. The binding affinity of 11q bound to WNV was also higher than that of SYC–1307 bound to the ZIKV protease. Due to its higher binding affinity and excellent bioavailability, 11q is likely to act as a pan-antiviral against DENV, WNV, and ZIKV infections. However, biochemical evaluations of protease activities in the presence of 11q are necessary to gain more insights into its inhibitory potential.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

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Ethics approval and consent to participate

Not applicable.

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Availability of data

All the data are provided in the manuscript. Raw files can be obtained from the corresponding author on request.

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ACCSCIENCE PUBLISHING

Gene & Protein in Disease

SHORT COMMUNICATION

B-cell lymphoma/leukemia 11A transcriptional targets and chromatin binding patterns in human leukemias

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Abstract

B-cell lymphoma/leukemia 11A (BCL11A) is a zinc-finger transcription factor that plays a crucial role in B-cell development. It is highly expressed in numerous neoplasias, making it a potential risk factor for cancer. Recently, we identified a subset of plasmacytoid dendritic cells (pDC) in mice that are derived from common lymphoid progenitors, which primarily give rise to lymphocytes. We further demonstrated that transcription of B cell-derived pDC (B-pDC) genes in this murine subset is highly regulated by BCL11A. To investigate whether a similar lineage exists in humans, we identified direct BCL11A transcription targets and chromatin binding patterns shared between malignant human pDC and B-cell leukemias. We focused on cell lines such as NALM6 (pre-B leukemia), Raji (B-cell Burkitt's lymphoma), and GM12878 (pre-B-cell leukemia) and compared BCL11A targets to those in the CAL-1 human pDC cell line. Our findings revealed that BCL11A bound to promoter regions of genes such as *PAX5*, *TCF3*, and *ID3* in B-cell leukemias, while it exclusively bound *AXL*, *SIGLEC1*, and *IGLL1* in CAL-1 human pDCs. These results suggest that an evolutionarily conserved transcriptional hierarchy underlies B-pDC commitment, distinguishing it in human leukemias.

Keywords: B-cell lymphoma/leukemia 11A; Transcription factor; Gene regulation; Leukemia; Chromatin immunoprecipitation sequencing analyses

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1. Introduction

Plasmacytoid dendritic cells (pDCs) were first characterized as a lineage derived from myeloid progenitors.[1,2] However, subsequent studies in both humans and mice raised questions about the true lineage affiliation of pDCs.[3-6] To address this issue, we previously employed B-cell-specific mb1(Cd79a/Igα)-driven Cre-mediated deletion in *Rosa26*-yellow fluorescent protein mice, which led to the identification of pDCs derived from common lymphoid progenitors (CLPs).[7,8] These pDC-like B lymphoid cells (B-pDCs) exhibited intrinsic activation, homed to secondary lymphoid organs, and, on expansion, promoted robust T-cell proliferation following toll-like receptor 9 engagement.[7] Further supporting this concept, Feng *et al.*[9] used a clonal lineage tracing strategy in mice and demonstrated a shared, FMS-like tyrosine kinase 3-dependent pathway for pDCs and B cells, originating from a shared CD81hi progenitor. Although B-pDCs do not secrete interferon-alpha, they express a unique profile of cytokines and exhibit high expression

of the membrane-associated AXL receptor kinase.[7] In that regard, B-pDCs phenotypically and functionally resemble previously characterized human AXL$^+$ DCs.[9-12]

To further investigate the presence of a B-pDC subset in human neoplasia, we performed transcriptional analyses on several human malignant hematopoietic lines. Our findings revealed a conserved dependency on the B-cell lymphoma/leukemia 11A (BCL11A) transcription factor for their specification, with a partial overlap of BCL11A targets seen in pDCs and B cells in murine hematopoietic stem cells.[8]

The development of both classical pDCs and B lymphocytes is regulated by BCL11A.[8,13-15] Both lineages share several BCL11A-regulated genes.[8] BCL11A overexpression has been observed in multiple malignancies,[16-18] including murine leukemia.[19-21] To determine if a B-pDC lineage exists in humans, we utilized chromatin immunoprecipitation sequencing (ChIP-seq) to identify direct BCL11A transcription targets and chromatin binding patterns that are shared between human pDCs and B-cell leukemias.

2. Materials and methods

2.1. Data deposition

Data in this study were deposited and can be accessed through the following accession numbers: current ChIP-seq (GSE99019) as well as previously published data, including pre-B ChIP-seq (GSE52868) and CAL-1 ChIP-seq (GSE55043) in the gene expression omnibus database, and BCL11A ChIP-seq (ENCODE GM12878) in the Encyclopedia of DNA Elements repository.

2.2. Chromatin immunoprecipitation followed by chromatin immunoprecipitation sequencing

We previously described the details of the BCL11A ChIP assays, analyzing the ChIP-seq data using Illumina technology.[8] The human lines employed for BCL11A ChIP-seq included NALM6 (human pre-B leukemia) and Raji (human Burkitt's lymphoma line). These were compared to the ChIP-seq of the human CAL-1 pDC cell line and the ChIP-seq of GM12878 (human B lymphoblastoid leukemia), acquired from the Encyclopedia of DNA Elements consortium. We analyzed all peak scores ≥10, employing the previously established BCL11A consensus binding site GGAAgcTGAAA.[8]

2.3. Statistical analysis

We defined a target gene as any gene with binding sites that occur within 50 kb upstream to the 5' end, including any occurring introns. Benjamini–Hochberg association[22] and associated *q*-values were performed to eliminate false discovery rates.

3. Results

The transcriptional dependency of BCL11A in both pDCs and B cells, along with their documented overlapping expression, led us to hypothesize that B-pDCs and B lymphocytes share common overlapping transcriptional programs. To test this hypothesis, we performed ChIP-seq analyses to identify BCL11A target genes in human leukemias and compared these findings to BCL11A targets in the human pDC CAL-1 cell line.[23] We analyzed data from the NALM6 pre-B leukemia,[24] the Raji B-cell Burkitt's lymphoma,[25] and the GM12878 pre-B-cell leukemia[26] and compared them to the data from the CAL-1 human pDC cell line. An important feature of CAL-1, in addition to its classical pDC expression, was its upregulation of the AXL receptor kinase.[25,26] Furthermore, CAL-1 was derived from malignant pDCs that express high levels of BCL11A.[23,27,28]

The ChIP-seq analysis for BCL11A was executed and plotted as previously detailed.[8] Figure 1A illustrates the overlap of genes bound by BCL11A among the four leukemias. Peak scores ≥10 are shown for NALM6 (green), Raji (black), CAL-1 (blue), and GM12878 (red). Numbers within the outer circle indicate statistically significant targets for each cell line, whereas the inner circle numbers denote the overlap of these targets among the four leukemias. Figure 1B presents BCL11A binding within critical target genes as defined as chromatin targets within 50 kb up- or downstream of transcriptional start sites. Benjamini–Hochberg statistics[20] were performed to determine *q*-values and associated false discovery rates.

As shown in Figure 1A, the expression of several prototypic B-cell transcripts (e.g., *IGLL1*, *IGLL5*, and *SPIB*) in CAL-1 indicated a close relationship to both pDCs and AXL$^+$ transitional DCs. However, the distribution of BCL11A occupancy in CAL-1 closely resembled that seen in three human leukemias, with approximately one-quarter of CAL-1 targets being shared (Figure 1A). Notably, we observed that in all leukemias, BCL11A is bound to its own promoter.

In addition to the array of BCL11A leukemia-related targets shared by B cells and pDCs, we observed context-dependent binding in others. While BCL11A bound promoter regions of genes, such as *PAX5, TCF3*, and *ID3*, exclusively in B cells, it occupied distinct sites in CAL-1 cells (e.g., *AXL*, *SIGLEC1*, and *IGLL1*). Notably, some target genes exhibited binding at different promoter positions, as evidenced by non-overlapping peaks in *TCF4* and *IRF4* or in *SPIB* and *ID2* (Figure 1B).

Moreover, BCL11A did not bind directly to the *CD19* locus in CAL-1 pDCs, but promoter-associated *CD19* peaks were present in all B-cell lines. Conversely, *CD86*

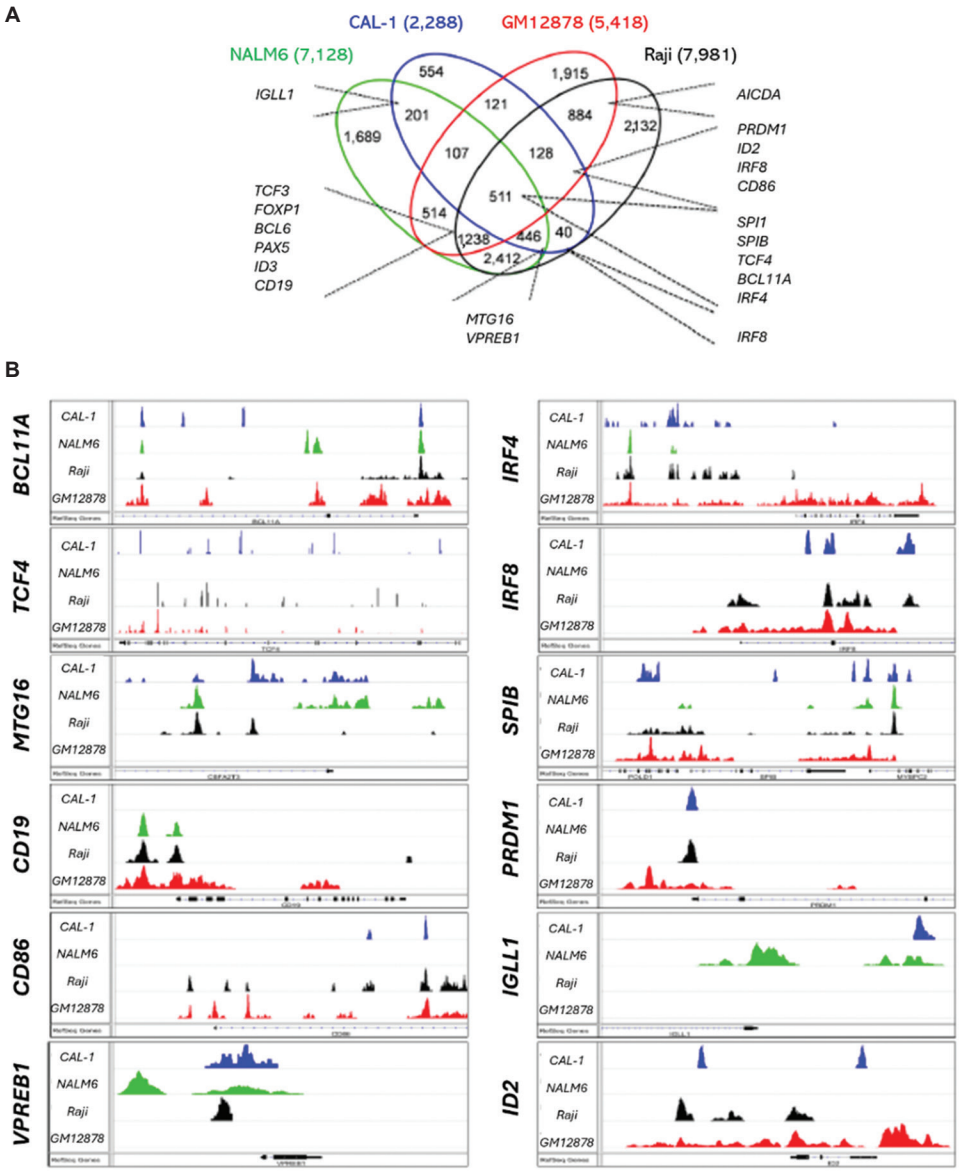

Figure 1. ChIP-seq analyses of genome-wide human BCL11A target binding in leukemias. ChIP-seq for *BCL11A* was performed in NALM6 (green) and Raji (black) and compared to ChIP-seq data acquired from the human pDC model CAL-1 (blue) and the Encyclopedia of DNA Elements consortium acquired ChIP-seq for B lymphoblastoid GM12878 (red, all peak scores ≥10). (A) Overlap of BCL11A target genes across the four human leukemias. Target genes were defined by a binding site occurring within 50 kb upstream through its intron containing the previously established[8] pDC binding consensus GGAAgcTGAAA. False discovery rate and associated *q*-values were calculated using Benjamini–Hochberg statistics. (B) Overlapping targets in B-cell and pDC leukemia targets unique to B-cell development and function were derived and highlighted. Some targets unique to B cells had peaks in CAL-1 pDC (i.e., *IGLL1* and *VPREB1*, peak scores ≥10).
Abbreviations: BCL11A: B-cell lymphoma/leukemia 11A; ChIP-seq: Chromatin immunoprecipitation sequencing; pDC: Plasmacytoid dendritic cells.

was bound by BCL11A in both mature B cells and CAL-1 pDCs. Intriguingly, in CAL-1 pDCs, BCL11A bound upstream of *IGLL1/Lambda-5* and *VPREB1* – genes expressed in mouse B-pDCs[7] – but not in myeloid-derived pDCs (Figure 1B). While our analyses identified BCL11A downstream targets, Runt-related transcription factor 2 directly regulates the transcription of BCL11A in acute myeloid leukemia.[29-31]

Previously, we identified in mice[7,8] and others in humans,[10-12] through single-cell RNA sequencing, a pDC with high expression of *AXL*, *SIGLEC1*, and *TCF4* (a costimulator of CD86) that produces lower levels of interferon-alpha on stimulation compared to its conventional pDC counterpart. Consequently, we analyzed the expression of these same defining genes in the human B-cell leukemias studied in this report. As shown

in Figure 1B, these genes were also highly expressed in the leukemias analyzed here, with the exception of *AXL*. The observation that *AXL* is targeted by BCL11A in the leukemias but not in CAL-1[32] pDC controls suggests that the well-documented AXL immunosuppressive function[33] might be lost in the leukemias. Together, these data further support the existence of B-pDCs in both mice and humans.

4. Discussion

We previously observed that, when overexpressed in conventional pDCs, BCL11A directly upregulated *ID3* and indirectly downregulated *ID2*.[8] The high expression of *ID2* in B-pDCs raises the likelihood that B-pDCs rely on a distinct developmental pathway. The reliance on B-pDC development on established B-cell transcription factors (including *ID2*, *ID3*, *TCF4*, and *BCL11A*) adds complexity that challenges a linear model of pDC specification.

In Figure 2, we present a revised model for pDC development that incorporates preexisting data as well as findings from this study and prior work by us and others.[7,8,32,34,35] This model (discussed in detail in the legend to Figure 2) proposes developmental routes originating from both the common dendritic progenitor and the CLP – both under BCL11A transcriptional control. We hypothesize that as CLP progenitors proceed to pre-pro B cells, a subset of primed B cells diverges to form the spleen-homing, intrinsically active, *AXL*$^{+}$*SIGLEC1*$^{+}$*CD19*$^{-}$ B-pDC subset described previously.[7]

Given the emerging clinical importance of AXL-mediated immunosuppression, it is unfortunate that a detailed understanding of its transcriptional regulation remains largely unknown.[36] While further studies are required, we propose adding BCL11A, at least in leukemias, to the short list of AXL transcriptional regulators. This hypothesis aligns with our previous observation of AXL

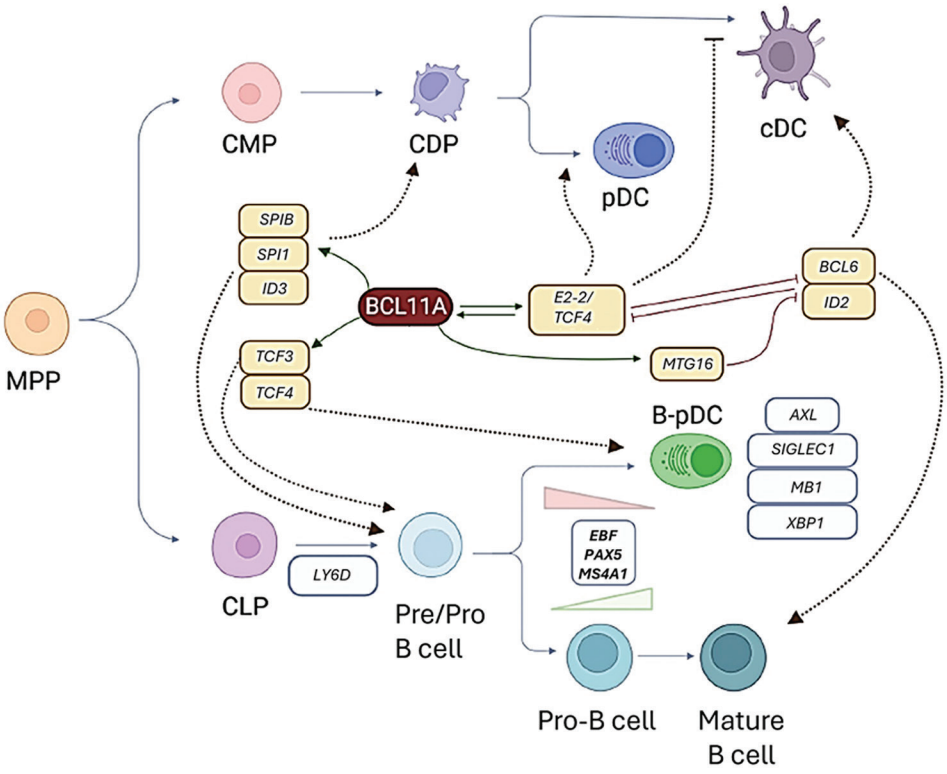

Figure 2. A detailed model of pDC and B-cell derived pDC development. We propose a two-pronged model for pDC differentiation. Following the split from multipotent progenitors (MPPs), common dendritic progenitors (CDPs) progress to pDCs by default through constitutive expression of BCL11A and TCF4 through a feedback loop. In this loop, BCL11A activates TCF4 and ID3 transcription, which, in turn, undergo heterodimerization, leading to a reduction in the activity of TCF4 (or other E-protein family members). In this way, ID3 (and perhaps BCL11A autoregulation) provides homeostatic maintenance of pDC by dampening TCF4. In the absence of BCL11A, conventional DC (cDC) persists, and an alternate CMP-CDP-cDC pathway is favored. Additional direct targets of BCL11A necessary for pDC development include *PU.1/SPI1* and *SPIB*. Second, pDCs can be alternatively generated from the CLP via an incompletely defined mechanism. These pDCs express increased levels of major histocompatibility class II, possibly under the direction of ID2 suppression of TCF3, an established promoter of B-cell development, and SPIC suppression of B-cell development (dotted grey lines).
Abbreviations: BCL11A: B-cell lymphoma/leukemia 11A; ID3: Inhibitor of differentiation 3; pDC: Plasmacytoid dendritic cells; SPIC: Spi-C transcription factor; TCF: Transcription factor.

immunostaining of B-pDCs.[7] Similar to BCL11A, AXL is upregulated by type I interferon to enhance its functional activity.[37]

We are confident that our data support the limited conclusions drawn. However, one experimental control remains missing, which is formal proof that the peaks observed in Figure 1B are exclusively dependent on BCL11A. To address this, we plan to generate clustered regularly interspaced short palindromic repeats knockout cell lines expressing critical BCL11A target genes and test whether the ChIP signal is eliminated on BCL11A knockout.

While this approach will require several months to execute properly, it is central to extending this work to bona fide human leukemic targets. Future efforts will focus on functional analyses of B-pDCs to better understand their role in immune response and cancer progression.

Another strategy involves reanalyzing our data using model-based analysis of ChIP-seq to identify additional mouse leukemic BCL11A target genes and overlapping binding sites within those genes. Despite potential limitations in quantitative accuracy, we remain confident that the major peaks identified were sufficient to support our conclusions. These findings, along with previously published data from others, form the foundation for the model presented in Figure 2.

Finally, further analyses into the specific transcriptional mechanisms downstream of these and additional target genes are required. This will help distinguish the implications of target cell-dependent differences in their regulation.

5. Conclusion

In our previous research and in this manuscript, we demonstrated that BCL11A not only regulates the development of classical pDCs and B-pDCs in mice, but that its overexpression also regulates murine B-cell leukemias in humans. Using ChIP-seq analyses, we showed that BCL11A exerts this regulatory effect through direct binding to the promoters and enhancers of genes critical to leukemia development. Further investigation into the specific transcriptional mechanisms downstream of these and other target genes is needed to clarify the impact of target cell-dependent differences in their regulation.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization: Joseph D. Dekker, Haley O. Tucker
Investigation: All authors
Methodology: All authors
Writing – original draft: All authors
Writing – review & editing: All authors

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

GSE105827 (RNA-seq), GSE99019 (ChIP-seq), and GSE52868 (pre-B RNA-seq) are the accession numbers to previously published datasets. Other data will be made available on request to the corresponding author.

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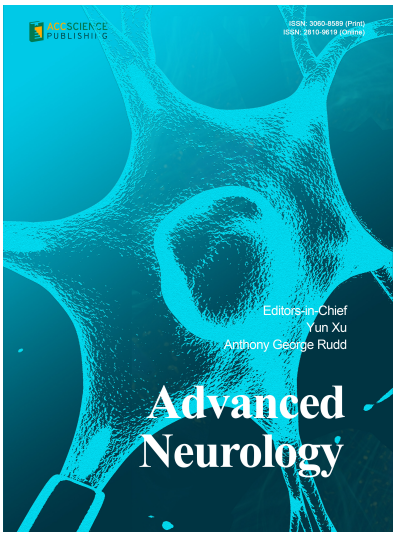

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