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Transient prenatal ruxolitinib treatment promotes neurogenesis and suppresses astrogliogenesis during embryonic mouse brain development

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Abstract: Ruxolitinib is a Janus kinase (JAK) inhibitor that inhibits the JAK/STAT signalling pathway by targeting JAK1 and JAK2, which are crucial for regulating astrogliogenesis. This study assessed the effect of ruxolitinib (5 and 30 mg/kg/day) on developing mouse brains by administering it to pregnant mice from E7.5 to E20.5. No adverse effects were observed in the treated mice. The brains of P1.5 pups were collected, and RNA was extracted to assess markers of neurogenesis and astrogliogenesis through RT-qPCR. The results revealed a significant decrease in *Gfap* expression (p<0.0001) in both ruxolitinib-treated groups compared to the control, indicating a suppression of astrogliogenesis. Additionally, *S100β* expression (p<0.05) was significantly reduced in the 30 mg/kg ruxolitinib-treated group. In contrast, the expression of neuronal markers *vGLuT1* (p<0.01) and *vGLuT2* (p<0.01) increased significantly in the 30 mg/kg treated group, suggesting enhanced neuronal differentiation. Furthermore, 5 and 30 mg/kg ruxolitinib-treated groups showed a significant increase in *GAT1* expression (p<0.01) compared to the control group. A marked decrease in *Nestin* expression was also observed in the 5 mg/kg (p<0.001) and 30 mg/kg (p<0.0001) treated groups. These findings demonstrate that transplacental administration of ruxolitinib modulates key markers involved in neuronal differentiation and gliogenesis in the developing mouse brain, suggesting its potential use in correcting imbalances in early brain development.

Keywords: Ruxolitinib; JAK-STAT pathway; Astrogliogenesis; Neurogenesis; Down syndrome.

1.0 INTRODUCTION

Ruxolitinib is a United States Food and Drug Administration (FDA)-approved kinase inhibitor that modulates the JAK-STAT signalling pathway. This pathway is essential for cellular differentiation and proliferation, especially in gliogenesis, where cytokines, hormones, and growth factors activate JAK through phosphorylation. Upon activation, JAKs phosphorylate STATs, resulting in their dimerisation and subsequent translocation into the nucleus, facilitating astrogliogenesis and inhibiting neurogenesis (Lee et al., 2016). The sustained activation of this pathway is linked to the enhancement of astroglial cell commitment, which detracts from neuronal development, a phenomenon known as the neurogenic-to-gliogenic shift (Dierssen et al., 2012).

Recent studies indicate that ruxolitinib may effectively inhibit this shift. Ruxolitinib targets JAK1/JAK2, leading to a reduction in astrogliogenesis, as demonstrated by its ability to suppress the expression of the astrocytic marker glial fibrillary acidic protein (GFAP) in the developing mouse brain (Lee et al., 2021). In addition to its anti-gliogenic properties, ruxolitinib has been demonstrated to influence tumour cells by inhibiting phosphorylated JAK2 (p-JAK2) and phosphorylated STAT3 (p-STAT3), indicating its potential as a modulator of cellular pathways (Wörmann et al., 2016). Furthermore, a different study has shown a decrease in the expression level of p-STAT3 in human non-small cell lung carcinoma cells following treatment with ruxolitinib (Looyenga et al., 2012).

Ruxolitinib's capacity to traverse the placental barrier is notably significant, as evidenced by a previous study indicating that oral administration of the drug at non-toxic doses during gestation had considerable impacts on the neural development of mouse embryos (Lee et al., 2021). The drug influences brain development by reducing astrocytic markers and enhancing cognitive function during early life stages, suggesting its potential to modulate essential developmental processes in the embryonic brain. Therefore, we aimed to evaluate the effect of ruxolitinib on the neural development of developing mouse brains, as it serves as a proof of concept for reversing the transition from neurogenesis-to-gliogenesis that is observed in Down syndromes (DS).

2.0 MATERIALS AND METHODS

2.1 Experimental Mice

The Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) authorised the guidelines and regulations governing handling all the mice used in this work and the methods, experiments, and tissue procurement. These guidelines were approved under reference number UPM/IACUC/AUP-R086/2015. The study adhered to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) criteria. The mice were housed in a controlled environment at the Genetics and Regenerative Medicine Research Centre (GRMRC), UPM. They were kept in individually ventilated cages with sterile airflow and maintained at a consistent temperature of 25°C. The mice followed a 12:12 h light-dark cycle in the mouse room facility. The mice had unlimited access to regular animal feed (Altromin 1324) and clean water. The animals were appropriately restrained throughout the treatment using a cappy cone or securely grasping their neck's scruff to ensure proper immobilisation.

2.2 Ruxolitinib Preparation and Treatment

Ruxolitinib (MedChem Express LLC, US) was dissolved in dimethyl sulfoxide (DMSO) at 60 mg/ml concentration as a stock solution and stored at -20°C before use. The stock solution was vortexed before mixing with 1% (w/v) methylcellulose (Amresco) in saline, followed by administrating it orally. The dosage used in this study was determined based on the non-maternally toxic dosages previously documented in the FDA report. Three groups of C57BL/6 aged postnatal day (P) 56–70 female mice, with five mice per group. These female mice were mated with stud males overnight. The vaginal plugs were examined the following morning to determine if successful mating. This day was designated as gestational or embryonic day (E) 0.5. Between E7.5 and E20.5, a control group (G1) was given a daily oral dose of 1% (w/v) methylcellulose in saline as a vehicle. Meanwhile, two more groups were administered ruxolitinib in methylcellulose daily, each receiving a different dosage: 5 mg/kg (G2) and 30 mg/kg (G3) based on body weight. Substances were administered orally or through intragastric gavage using a 22G steel gavage with a ball tip (Harvard Apparatus). Following birth, the P1.5 pups were weighed, then anaesthetised on an ice-cold petri dish and subsequently decapitated. The P1.5 brain was harvested in ice-cold phosphate-buffered saline (PBS) and kept in TRIzol (Invitrogen) solution for RNA extraction.

2.3 RNA Extraction and cDNA Synthesis

RNA was extracted from the whole brain using TRIzol, following the manufacturer's protocol. Subsequently, the RNA was dissolved in DEPC-H2O (Invitrogen) as an elution buffer. A total of 2 µg of RNA was synthesised using the SuperScript IV Reverse Transcriptase kit. The

RNA synthesis procedures adhered to the manufacturer's protocol.

2.4 RT-qPCR Analysis

The RT-qPCR (QuantiTect SYBR Green PCR kit, Qiagen) was performed in a 10 µl total reaction to identify several markers of stem cell (*Oct4*, *Nanog*, and *Sox2*), neural progenitor (*Sox1* and *Nestin*), pro-neural (*NeuroD1*), neuronal cell (*GAT1*, *vGluT1*, and *vGluT2*) and astrocyte (*Gfap* and *S100β*). Each reaction consists of 0.3× of cDNA, 1 µM forward and reverse primers, 1× QuantiTect master mix, and an appropriate amount of DEPC-H_2O. The PCR cycle was set up for 40 cycles of 94°C for 10 seconds, then 50-60°C for 30 seconds, and then 72°C for 10 seconds. A minimum 4-data point standard curve was constructed using serially diluted pooled cDNAs for each primer set used in qPCR in each run. The standard curve was used to determine the PCR efficiency and reproducibility of each PCR system. The housekeeping genes *Rpl13a*, *Rpl27*, and *Tbp* were used as reference genes. The Second Derivative Maximum method determined each signal's crossing point (Cp) (Luu-The et al., 2005). A series of diluted cDNAs was utilised to create a standard curve consisting of four data points for each PCR system. Three reference genes were utilised as endogenous controls. The reference gene Cp values were used to calculate the geometric mean, which was then normalised with the target gene to estimate the starting amount of each gene. A linear model was applied to analyse each gene's time course of expression values. **Table 1** depicts the list of primers involved in this continuous study and their properties.

Table 1. List of primers and their properties.

Markers	Gene		Primer (5'→3')	GC%	Tm (°)	Amplicon size
Stem Cell	*Nanog*	Forward	TGCTTACAAGGGTCTGCTACTG	50	51	76
		Reverse	GAGGCAGGTCTTCAGAGGAA	55		
	Oct4	Forward	CACGAGTGGAAA GCAACTCA	50	51	129
		Reverse	GCTTTCATGTCC TGGGACTC	55		
	Sox2	Forward	CGCCCAGTAGAC TGCACA	61	51	95
		Reverse	CCCTCACATGTG CGACAG	61		
Neural Progenitor	*Sox1*	Forward	TTGAGGCAGCTGGGTCTC	61	55	75
		Reverse	GCTGTTGTCCCTATCCTTGG	55		
	Nestin	Forward	TCCCTTAGTCTGGAAGTGGTCA	50	55	68
		Reverse	GGTGTCTGCAAGCGAGAGTT	55		
Pro-Neural	*NeuroD1*	Forward	GGGAACAGCCTTACCCTTGT	55	53	67
		Reverse	CCACCAGAAATCACCAGGAG	55		
Astrocyte	*Gfap*	Forward	CGCCACCTACAGGAAATTG	53	60	76
		Reverse	CTGGAGGTTGGAGAAAGTCTGT	50		
	S100β	Forward	TGAAGGAGCTTATCAACAACGA	41	60	79
		Reverse	TCCATCACTTTGTCCACCAC	50		
Neuronal	*GAT1*	Forward	GCCTGGTCAATACCACCAAC	55	60	76
		Reverse	CCATCTGTCATCTGGTGCAT	50		
	vGluT1	Forward	GTGCAATGACCAAGCACAAG	50	60	84
		Reverse	AGATGACACCGC CGTAGTG	58		
	vGluT2	Forward	GGAAAATCCCTC GGACAGA	53	60	61
		Reverse	TGGTCTCTCGGTT GTCCTG	58		
Housekeeping Genes	*Rpl13a*	Forward	AGCCTACCAGAAAGTTTGCTTAC	43	58	129
		Reverse	GCTTCTTCTTCCGATAGTGCATC	48		
	Rpl27	Forward	AAGCCGTCATCGTGAAGAACA	48	58	143
		Reverse	CTTGATCTTGGATCGCTTGGC	52		
	Tbp	Forward	CCTTGTACCCTTCACCAATGAC	50	58	119
		Reverse	ACAGCCAAGATTCACGGTAGA	48		

2.5 Statistical Analysis

All comparisons were based on one-way ANOVA with Tukey's post-hoc test (n=3 per group). Statistical analyses were performed using GraphPad Prism 9.3.1 software. Differences between control and treatment groups were considered significant at *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

3.0 RESULTS AND DISCUSSION

This study administered the established safe dose of

ruxolitinib (30 mg/kg/day as reported in Lee et al., 2021) to assess its impact on suppressing astrogliogenesis and its capacity to enhance neurogenesis in the developing mouse brain. Further analysis was performed by analysing several markers of stem cells, neural progenitor, pro-neural, neuronal cells, and astrocytes and comparing their expressions between the treated and control groups (**Figure 1**).

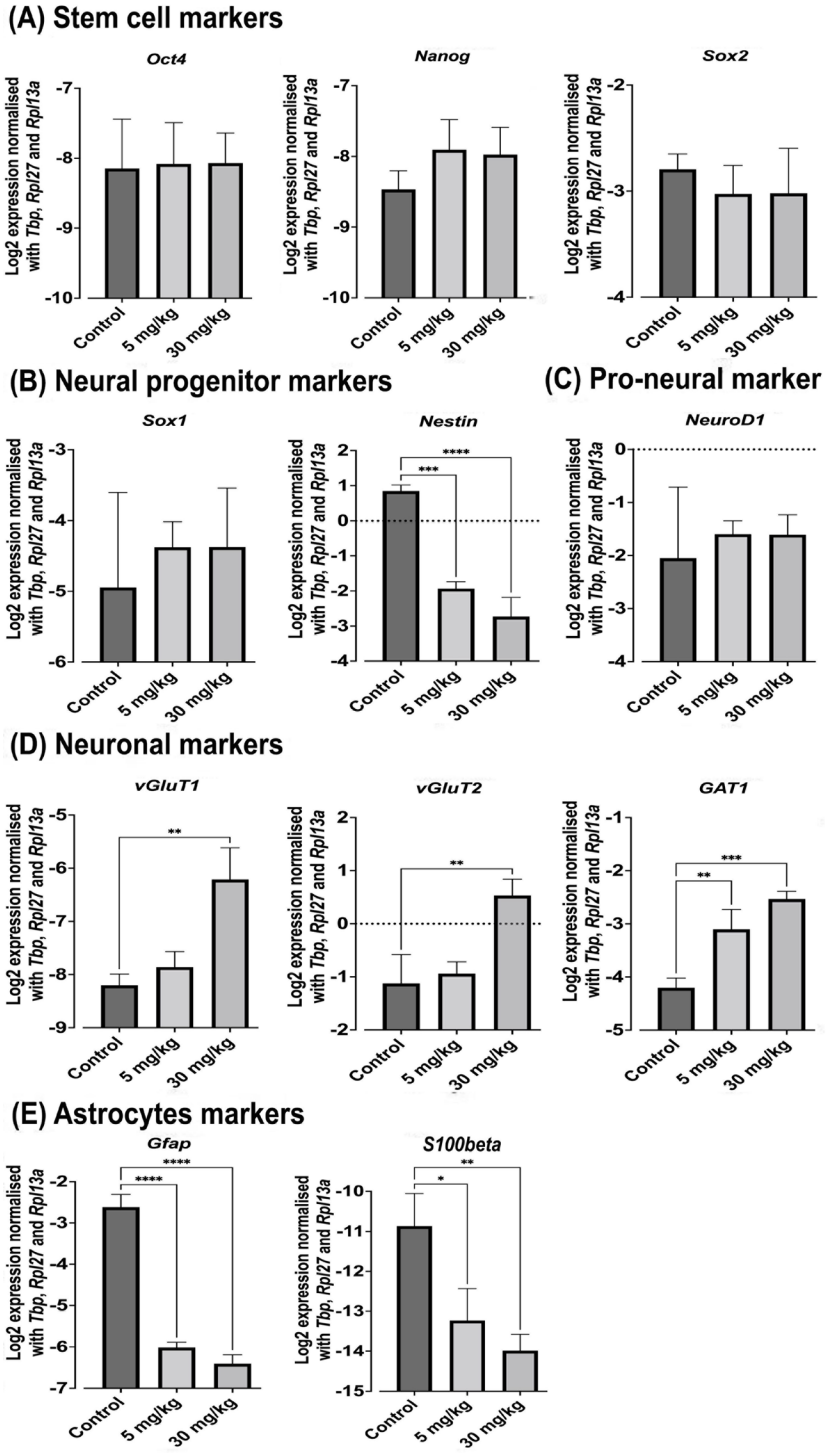

Figure 1. Gene expression profile of the gliogenic-suppression effect of ruxolitinib in the postnatal brain. Various (A) stem cells, (B) neural progenitor, (C) pro-neural, (D) neuronal, and (E) astrocyte markers were assessed. Values are presented as mean ± *SD*. Statistical analysis was performed using one-way ANOVA with Tukey correction. The asterisk denotes significant differences between the 30 mg/kg group and the control at $p<0.05$(*), $p<0.01$(**), $p<0.001$(***), and $p<0.0001$(****); $n=3$ biological replicates.

Statistical analysis showed that the neural progenitor marker *Nestin* significantly decreased in the 5 mg/kg group (p<0.001) and the 30 mg/kg group (p<0.0001). Furthermore, the expression of *vGluT1* and *vGluT2*, a neuron marker, was elevated significantly in the group treated with a 30 mg/kg dosage (p<0.01). Intriguingly, *GAT1* exhibited a significant upregulation in both groups, 5 mg/kg (p<0.01) and 30 mg/kg (p<0.001) compared to the control. Conversely, the expression of astrocyte markers was decreased, particularly *Gfap*, which was significantly reduced (p<0.0001) in the 5 and 30 mg/kg groups compared to the control. Similarly, *S100β* was significantly downregulated in both groups, 5 mg/kg (p<0.05) and 30 mg/kg (p<0.01) compared to the control. While stem cell markers (*Oct4*, *Nanog*, and *Sox2*), *Sox1*, and *NeuroD1* showed no significant difference compared to the control. The findings indicated that administration of ruxolitinib at 5 and 30 mg/kg effectively suppressed astrogliogenesis by inhibiting the JAK kinase domain. Our findings aligned with a previous study, demonstrating a significant decrease in *Gfap* expression in the mouse brain in the groups treated with ruxolitinib (Lee et al., 2021).

GFAP plays an essential role in astrocyte maturation and reactivity, particularly during brain development, where astrocytes contribute to synaptic support, neuronal signalling, and blood-brain barrier integrity (Abbink et al., 2019). S100β, a calcium-binding protein, is involved in calcium signalling, neuroinflammation, and astrocyte function (Michetti et al., 2023). The reduction in *Gfap* expression suggests that ruxolitinib may decrease astrocyte reactivity or modify the structural state of astrocytes, potentially leading to a less reactive or more quiescent astrocytic phenotype. The slight reduction in *S100β* expression indicates that ruxolitinib might also impact calcium-mediated signalling and neuroinflammatory processes in astrocytes, albeit to a lesser extent than its impact on *Gfap*. Ruxolitinib, a JAK1/JAK2 inhibitor, appears to reduce the overall reactive state of astrocytes by inhibiting the JAK/STAT pathway, as evidenced by decreased expression of both *Gfap* and *S100β*. Moreover, previous studies have documented that ruxolitinib decreases GFAP expression by up to 60% in mouse brains (Haile et al., 2016). In line with these findings, our study demonstrated that ruxolitinib exerts a transplacental suppressive effect on astrogliogenesis during in-utero development by downregulating both *Gfap* and *S100β*.

In addition, *Nestin* was also found to be downregulated in the ruxolitinib-treated group. Nestin is a cytoskeletal protein classified as an intermediate filament and co-polymerisation partner that forms heteropolymers in astrocytes, together with vimentin, GFAP, synemin and lamins (Eliasson et al., 1999; Pekny et al., 1999; Bellin et al., 1999; Steinert et al., 1999). Nestin expression was significantly elevated in differentiated trisomic-iPSC (T21-iPSC) derived from DS patients (Mollo et al., 2021). Additionally, this study revealed the overexpression of several glial markers, including GFAP and S100β, in T21-iPSC compared to the control. Our understanding of the roles of Nestin in astrocytes remains limited. Nestin was expressed in immature and primary astrocyte cultures (Puschmann et al., 2013; Pekny et al., 1998). While in vivo, Nestin was strongly expressed only by a subset of GFAP+ cells. Approximately 30% of the Nestin-expressing cells in the dentate gyrus (DG) are astrocytes (Filippov et al., 2003). Moreover, the distribution of Nestin in astrocytes showed that Nestin was primarily found in cell protrusion (Thomsen et al., 2013). This indicates that Nestin plays a crucial role in the reorganisation of astrocyte morphology. Thus, inhibiting astrogliogenesis not only affects the levels of *Gfap* and *S100β* but also decreases the expression of *Nestin*.

In this study, treatment with ruxolitinib elevates the expression of neuronal markers (*vGluT1*, *vGluT2*, and *GAT1*). These findings aligned with a previous study, in which the expression of *Tuj1* and *NeuN* (neuronal markers) were upregulated in P1.5 whole-brain mouse, whereas the P1.5 whole-brain and neurosphere protein lysates and demonstrated that the levels of Tuj1 expression remained unchanged in the ruxolitinib-treated group (Lee et al., 2021). Ruxolitinib inhibits JAK1 and JAK2, which are crucial for the JAK-STAT signalling pathway and are essential for astrogliogenesis. By binding to the ATP-binding sites of JAK1 and JAK2, ruxolitinib prevents their activation and subsequent phosphorylation of downstream targets, including STAT3. Without active STAT3, astrocyte-specific genes like *Gfap* and *S100β* are not transcribed, inhibiting the differentiation of neural progenitor cells into astrocytes (**Figure 2**). Our findings also show that the administration of ruxolitinib inhibits the expression of these specific astrocyte markers and impacts *Nestin*. Nevertheless, the relationship between *Nestin* and its function in facilitating astrogliogenesis remains insufficiently studied. Simultaneously with the suppression of astrogliogenesis, neural stem or progenitor cells were promoted into neurogenesis.

These findings show the transplacental astrogenesis-suppression effect on the developing embryonic mouse brain. Furthermore, ruxolitinib may, besides inhibit

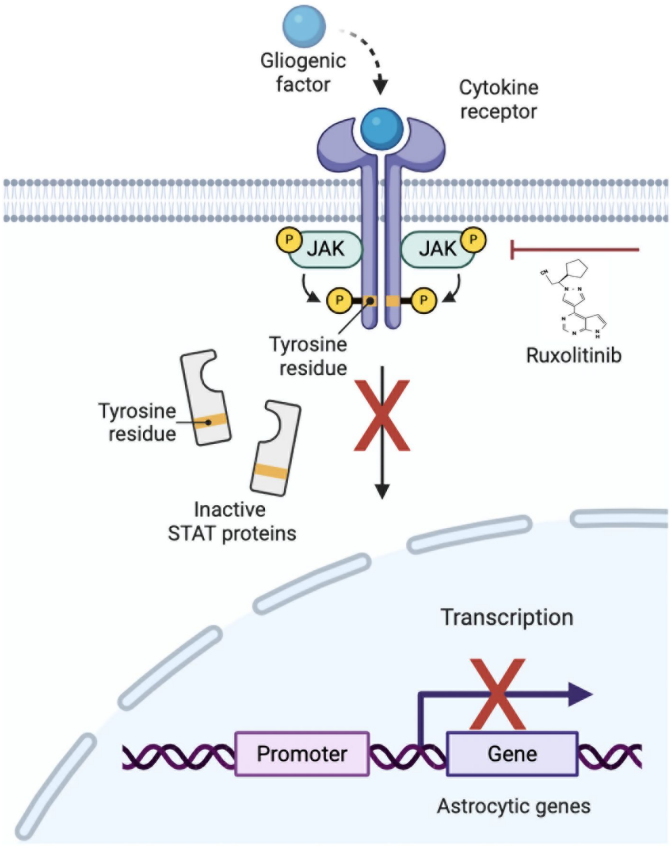

Figure 2. Ruxolitinib competitively binds to the catalytic region in the kinase domain, directly inhibiting JAK1-2 by competing with ATP for binding. Ruxolitinib suppresses the JAK-STAT signalling pathway, significantly reducing astrogliogenesis (*Gfap* and *S100β*), and simultaneously promoting neuronal differentiation.

astrogliogenesis yet might also contribute to the reduction of inflammation in the DS brain, thereby alleviating reactive gliosis and fostering a more favorable environment for neuronal development and differentiation. Further fundamental investigations on how early suppression could affect the brain's postnatal neuroplasticity throughout maturation and ageing should be evaluated. Nonetheless, the study has given substantial proof of ruxolitinib's potential use in neurological disorders, which must be proceeded cautiously.

4.0 CONCLUSIONS

In summary, we have examined the expression of several markers in the developing brain of mice after administering ruxolitinib. Our findings showed that ruxolitinib treatment may suppress astrogliogenesis and promote neurogenesis indirectly via the modulation of the JAK/STAT signalling pathway during brain development. Interestingly, ruxolitinib has been found to influence brain progenitor/neural stem cells, although there were no statistically significant differences between the control and treated groups. This is attributed to the constrained sample size, which may impact mathematical analysis. Therefore, further studies are necessary to decipher the impact of ruxolitinib on brain development.

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Conflicts of Interest: The authors declare no conflict of interest.

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