UNIVERSITI PUTRA MALAYSIA

EFFECT OF RUXOLITINIB SUPPLEMENTATION IN ENHANCING NEUROGENESIS VIA GLIOGENESIS SUPPRESSION IN FOETAL MOUSE BRAIN DEVELOPMENT

HAMIZUN BIN HAMZAH

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2019

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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May 2019

Supervisor : Michael Ling King Hwa, PhD
Faculty : **Medicine and Health Sciences**

JAK-STAT pathway is important in regulating gliogenesis in the brain. Dysregulation of this pathway leads to abnormality in brain development as seen in Down syndrome individuals' predominantly low initial neuron-glia ratio in early infancy. Thus, suppression of JAK-STAT pathway would be advantageous to reverse gliogenesis towards potentially enhance neurogenesis of which may serve as an essential building block for intellectual capability in Down syndrome individuals. To date, there is only one United State Food and Drug Administration (FDA) approved drug that targets both JAK1 and JAK2 proteins. This study aims to evaluate the toxicity and ability of ruxolitinib to suppress JAK-STAT pathway in the mouse brain when being supplemented for a long term at no observed adverse effect level (NOAEL) doses of 30mg per kilogram body-weight and below. Coherently, a control group of pregnant mice fed with methylcellulose as the vehicle and another five groups of pregnant mice were treated daily with different doses of ruxolitinib (1mg, 5mg, 10mg, 15mg and 30mg per kilogram body weight) dissolved in methylcellulose via oral administration during pregnancy E7.5 through E21.5 before delivery. At P1.5 post-delivery, multiple organs were harvested from mothers such as blood, liver, kidney and spleen for toxicity screening, whereas pup whole brains were dissected for JAK proteins analysis and gene expression. Inherently, blood biochemistry showed a normal reading on liver and kidney analytes, while histology observation revealed normal cellular morphology without discernible lymphocyte infiltration in these organs thus establishing the drug, ruxolitinib is non-toxic for a long-term administration on the pregnant mouse when administering at NOAEL doses. Subsequently, western blot analysis of the P1.5 brain lysates showed no significant differences in beta-tubulin III (Tuj1) for neuronal cells in all treated groups when compared to the untreated group. However, a significant reduction in glial fibrillary acidic

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protein (GFAP) for glial cells was observed at 30mg/kg ruxolitinib-treated group. However, none of the phosphorylated JAK1 and JAK2 were seen reduced in P1.5 brain suggesting JAK-STAT signalling pathway may not have been effectively targeted. The findings are subjected to further analyses, and when comprehensively validated, the application of this study would institute prepregnancy prescription of ruxolitinib as supplementation to expecting mothers of late maternal age who are at high risk of having a baby with developmental disorders such as Down syndrome.

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KESAN SUPLEMENTASI RUXOLITINIB UNTUK MERANGSANG NEUROGENESIS MELALUI PENINDASAN GLIOGENESIS SEMASA PERKEMBANGAN OTAK FETUS MENCIT

Oleh

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Laluan JAK-STAT memainkan peranan penting di dalam mengawalatur perkembangan gliogenesis di dalam otak. Ketidakaturan laluan ini boleh menyebabkan keabnormalan perkembangan otak seperti yang berlaku ke atas penghidap sindrom Down di mana jumlah kadar nisbah neuron dan glia yang rendah di awal peringkat umur bayi. Oleh itu, penindasan laluan JAK-STAT mungkin memberi kelebihan melalui penterbalikkan gliogenesis yang berpotensi meningkatkan neurogenesis dan seterusnya menjadi bahan asas untuk memperbaiki keupayaan intelek penghidap sindrom Down. Sehingga kini, hanya terdapat satu ubat yang diakui oleh *United State Food and Drug Administration* (FDA) yang mensasarkan kedua-dua protein JAK1 dan JAK2, iaitu ruxolitinib. Kajian ini bertujuan untuk menilai ketoksikan dan keupayaan ruxolitinib untuk menindas laluan JAK-STAT di dalam otak mencit selepas diberi supplementasi dalam tempoh yang panjang pada dos *no observed adversed effect level* (NOAEL) sebanyak 30mg per kilogram berat badan dan ke bawah. Dalam hubungan ini, satu kumpulan kawalan mencit dewasa yang bunting diberi makan hanya metilselulos sebagai perantara manakala lima lagi kumpulan diberi rawatan ruxolitinib yang dilarutkan di dalam metilselulos juga secara oral setiap hari mengikut dos ruxolitinib yang berbeza iaitu 1mg, 5mg, 10mg, 15mg dan 30mg per kilogram jisim badan mencit sepanjang tempoh kebuntingan E7.5 sehingga E21.5 sebelum melahirkan anak. Pada hari P1.5 selepas kelahiran, beberapa organ daripada ibu mencit diambil iaitu darah, hati, ginjal dan limpa untuk penentuan kesan toksik manakala otak anak mencit diambil untuk analisa protein dan ekspresi gen. Diperhatikan bahawa biokimia darah menunjukkan bacaan status hati dan ginjal mencit yang normal sementara pemerhatian histologi menampakkan morfologi sel yang normal tanpa kelainan infiltrasi limfosit yang seterusnya mengesahkan bahawa ruxolitinib adalah tidak toksik ke

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atas mencit hamil apabila diberi dos-dos NOAEL untuk tempoh jangkamasa yang panjang. Seterusnya, analisa *western* blot ke atas *lysate* otak anak mencit berumur P1.5 menunjukkan bahawa tiada perubahan ketara tahap protein penanda bagi sel saraf iaitu *beta-tubulin III* (Tuj1) dalam semua dos rawatan ruxolitinib. Akan tetapi, *glial fibrillary acidic protein* (GFAP), penanda bagi sel glia, didapati berkurangan dalam kumpulan yang dirawati dengan 30mg/kg ruxolitinib. Pada masa yang sama, tiada satu dos dapat menurunkan tahap fosforilasi protein JAK1 dan JAK2 dalam otak mencit P1.5 mencadangkan laluan isyarat JAK-STAT tidak dapat disasar secara efektif. Bergantung kepada analisa-analisa susulan lain, aplikasi kajian ini memungkinkan preskripsi ruxolitinib sebagai suplemen/makanan tambahan untuk tempoh sebelum kehamilan bagi wanita lanjut usia yang berisiko tinggi mendapat anak yang mempunyai masalah perkembangan seperti sindrom Down.

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Thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMP	Bone morphogenetic protein
E7.5 through E21	Age of embryonic foetus 7.5 until 21 days old
FGF	Fibroblast growth factor
GW	Gestational week
HSA21	Human Chromosome 21
IC50	50% inhibitory concentration
JAK	Janus associated kinase
MMU16	Mouse chromosome 16
NOAEL	No observed adverse effect level
P1.5	Age of mouse at 1.5 days old
P56-70	Age of mouse at 56-70 days old
SVZ	Subventricular zone
SGZ	Subgranular zone
STAT	Signal transducer and activation of transcription
SHH	Sonic Hedge Hog
Tyk2	Tyrosine kinase 2
USFDA	United States Food and Drug Administration
Wnt	Wingless/ Beta Catenine Interactive pathway

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CHAPTER 1

INTRODUCTION

1.1 Background

Every one of us is billionaires. The human brain, as the most vital organ, is made of approximately 100 billion neurons supported by 900 billion glial cells for it to function (Houzel S. H. 2009). Neurons have a distinct function which is to transmit impulse signals. On the other hand, glial cells that comprise oligodendrocytes, astrocytes, and ependymal cells play different roles to regulate the microenvironment of the brain to support and to protect neurons. Ancestral lineages generate both types of cells under the process called neurogenesis and gliogenesis, respectively.

Neurogenesis is a process of the birth of neurons from neural progenitor cells. Neurons are generated from early embryonic brain until early postnatal period, with limited neurogenic zones remaining active in the adult (Eriksson *et al.,* 1998) such as in the subventricular zone (SVZ) and the subgranular zone (SGZ) in foetal brain. Neuronal cell fate is determined by many regulatory pathways, namely Notch, Wnt, JAK-STAT. Notch signalling pathway serves essential functions in different developmental and homeostatic processes. It can promote tissue growth such as sensory hair cells and branched arterial networks as well as suppress cells on different occasions, causing tumour suppression (Sarah J. Bray, 2016). Wnt signalling is involved in controlling gene expression, cell adhesion, axon growth and cell proliferation. The focus in this study is to evaluate the inhibition of JAK-STAT signalling during foetal mouse brain development by using the United States Food and Drug Administration approved drug known as ruxolitinib at No Observed Adverse Effect Level (NOAEL) at 30mg per kilogram body weight and lower doses as a supplementation throughout pregnancy (FDA report from https://www.accessdata.fda.gov).

1.2 Janus Associated Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT)

Janus associated kinase (JAK) and signal transducer and activator of transcription (STAT) pathway is important signalling that takes part in the regulation of gliogenesis. The pathway involves the activation of JAK and STAT proteins leading to a cascading regulatory effect on gene expression in the central nervous system during foetal brain development, hormonal release,

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inflammation and tumorigenesis (Celine S. Nicolas *et al.*, 2013). Dysregulation of the JAK-STAT pathway was found as the underlying reasons in haematological malignancies such as myeloid disorders, namely myeloproliferative neoplasm and myeloid leukaemia (Furqan *et al*., 2013).

Janus associated kinase (JAK) is a family of intracellular tyrosine kinases. They play essential roles in directing cascading cellular response from the outside cell membrane into the regulation balance of cell growth, proliferation and differentiation, gene expression, cell survival, apoptosis and immune response against adverse antigens following binding of various ligands to the corresponding extracellular receptor (Babon *et al.,* 2014). JAK is one of ten recognised families of non-receptor tyrosine kinase where mammals have four consecutive proteins namely JAK1, JAK2, JAK3 and tyrosine kinase 2 (Tyk2) (Yamaoka *et al*., 2004). They are relatively large molecules with approximately 1,100 amino acids and molecular size 120-140kDa. On the other hand, STAT would best be described as latent transcription factor comprises of seven members (STAT 1, 2, 3, 4, 5A, 5B and 6). This unique combination of cytokines, receptor, JAK and STAT can regulate cellular response such as inducing more cytokines production to cascade other cells to react and/or proliferate (Jason S. Rawlings *et al.,* 2004).

Both JAKs and STATs work in tandem whenever specific antigen and/or cytokines bind to cellular transmembrane receptor hence activating specific JAK proteins following recruitment/activation of STAT. Initially, cytokine signalling is instigated through ligand interaction with its specific trans-membrane receptor subunits by which subsequent receptor oligomerisation would result in activation via phosphorylation of either an intrinsic kinase domain or receptor-associated JAK kinases. Then, JAKs will initiate a cascade of intracellular phosphorylation of various proteins including STATs (Willis X. Li, 2008).

Following JAK phosphorylation, it would activate STAT to dimerise and translocate into the nucleus where it starts to modulate gene expression. According to Villarino *et al*., 2017, the JAK-STAT signalling begins when extracellular cytokines react with their correspondent transmembrane receptor. The protein-bound complex then precipitates intracellularly which later phosphorylates the cytoplasmic tail of the receptor to create a suitable site for STAT activation and dimerisation. It will then translocates into the nucleus, attach to the specific gene sequence thence initiates gene transcription.

During foetal development, the germinal neural epithelium is actively differentiating and proliferating into neurons and glia and these activities are

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termed neurogenesis and gliogenesis. Normal cellular mechanism will follow the sequence where progenitor cells in neural tube differentiated into neurons and glia after receiving intrinsic and extrinsic factors from environ of developing foetus (M. Berry, 1986). Neurons are generated from progenitor cells with the help of Wnt or Beta-Catenin, Sonic Hedge Hog (SHH) and/or Notch signalling pathway (Juan J. Sanz-Ezquerro *et al*., 2017). Nevertheless, should any part of the cascade be dysregulated, neurogenesis will be affected. Gliogenesis, on the other hand, would depend mainly on the JAK-STAT signalling pathway, bone morphogenetic protein (BMP) and/or Notch signalling pathway to produce enough glia (Wen *et al*., 2009). A precise mechanism must take place through a concerted effort of different pathways to work precisely at the right time be it towards activation and/or repression of the signalling process.

Dysregulated pathway would induce disorder and disease such as hematologic disease, Huntington's disease and Down syndrome. Down syndrome, taking its name after a physician, John Langdon Haydon Down whom first described the similarities to that of mongoloid facial features in European parentage children and other distinctive characteristics in his paper, "Observations on an Ethnic Classification of Idiots" which was published in 1866. It was later found out by Jerome Lejeune, a French physician in 1959 who discovered the extra-chromosomal presence in the cell caused the condition. Karlsen *et al.* (2011) identified that there is an anomaly caused by the presence of an extra number of chromosome 21 (HSA21) which led to the disease. Tan *et al.,* (2014) and Ling *et al*., (2014) iterated the gene expression in Ts1Cje mouse model of Down syndrome showing neuropathological consequence were found to develop in the brain of mice having partial triplication of its chromosome 16 (MMU16: *Mus musculus 16*). Another astounding feature found by Karlsen *et al*. (2011) was that the total numbers of neurons and glia in the brain of Down syndrome adult were affected by delayed development as well as accelerated ageing which causes concomitant Alzheimer-like pathology having 40% lower number of neocortical neurons and 30% fewer neocortical glia while 50% oligodendrocyte reduction in the basal ganglia.

Nevertheless, proactive steps can be done to increase the neuronal number. A promising way would be manipulating the JAK-STAT signalling pathway by inhibiting gliogenesis using the drug during foetal brain development. Hence, the emergence of drugs able to exploit the activity of JAK-STAT would be useful for such an approach.

According to Mascarenhas *et al.* (2013), the ability of ruxolitinib to inhibit and downregulate the activity of JAK is anticipated to reduce the expression of STAT thus preventing it to translocate into the nucleus to regulate gene expression. An evaluation of 2 double-blinded, randomised, placebo-controlled studies showed

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changes in inflammatory markers in response to the therapy. Sun *et al*. (2003) observed that neural stem cells have extraordinary potential to repair the damaged nervous system, yet the multipotent and bipotent neural progenitor cells will lose their neurogenic potential after expansion. In due course, an increasing number of neurons means higher building blocks for neuronal networks formation and consolidation, the foundation of intellectual capability. Rusznak *et al*. (2016) reviewed that by modulating the balance on both neurogenesis and gliogenesis, it could pave a potential path towards neuro-restoration especially in neurogenesis and/or gliogenesis related diseases.

It is interesting that dysregulated JAK-STAT pathway is involved in neurogenic-to-gliogenic shift in the brain. JAK-STAT pathway dysregulation has been found in many disorders such as haematological malignancies (Furqan *et al*., 2013), while James C *et al.* (2005) described that a clonal and recurrent mutation in the JH2 pseudo-kinase domain of the JAK2 gene were found in polycythaemia patients. Malinge *et al.* (2004) also found that following their attempts for leukaemia screen for gene mutation of JAK2 which led them to the identification of JAK2-acquired mutation in a patient with Down syndrome. The finding highlights and indicates the involvement of the JAK-STAT pathway in children with Down syndrome. According to Watson-Scales *et al*. (2018) who relates that motor dysfunction in Down syndrome individuals was due to motor neuron degeneration citing on observation by Moldrich *et al.* (2007) where imbalance gene dosage was affecting cerebellum development. It was also mentioned by Pinter *et al*. (2011) that high-resolution MRI images showed smaller overall brain volumes with disproportionately smaller cerebellar volumes and relatively large subcortical grey matter volumes. Primarily during brain development in Down syndrome foetus, the underlying hedge, dysregulation of JAK-STAT signalling pathway leads to anomalous number of low initial neuron whilst its progenitor cells tend to commit to glial cell fate (gliogenesis) rather than transform into functional neurons (neurogenesis) as seen in Down syndrome individuals, a condition known as “neurogenic-to-gliogenic shift”. Based on the shift, neural progenitor cells of Down syndrome are expected to preferably commit into glia rather than neurons thus low initial neuron-glia ratio in the brain as opposed to the healthy brain (Houzel *et al.,* 2014). Yeo *et al*. (2004) elaborated the importance of programmed cell death which could relate to this study where a comparatively low number of neurons in Down syndrome individuals may have a higher pace of neurodegeneration in the brain thus potentially causes behavioural and memory disabilities.

This study delves into using ruxolitinib for its potent ability to inhibit JAK-STAT signalling pathway. The enhancement effect on neurogenesis via the inhibition of gliogenesis on normal developing mouse brain was studied. The study intends to evaluate its preliminary potential on a foetus and the mother for future expansion of use as a supplementation on high-risk mother carrying Down syndrome foetus.

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Nonetheless, by doing so, precautionary measures must be taken into consideration concerning safety both to the mother and foetus; also the method of administration, distribution, metabolism and excretion must be evaluated. This study, however, will contribute as one of the promising potential early intervention and remedy to revert the neurogenic-to-gliogenic shift while hoping to be able to increase the number of neurons in the foetal brain. The mouse model will be a good representative into observing the potential of oral supplementation towards a pregnant mouse impelling neurogenic stem cells from gliogenic progenitor cells to achieve an excellent high initial number of neurons present in the foetal brain. This will also be applicable onto human prepregnancy supplementation to increase intellectual ability towards a better life and delaying onset of degeneration of neurons to occur. Ruxolitinib is a potential supplement for neurogenesis enhancement because the introduction of Ruxolitinib as JAK-STAT inhibitor for its capability to act accordingly without the risk of toxicity makes it the best candidate for the hallmark suppressing gliogenesis in Down syndrome. Prior the United States Food and Drug Administration approval on 16 November 2011 (http://wayback.archive-it.org), ruxolitinib was tested at the Centre for Drug Evaluation and Research which concur 30mg/kg body weight as the safest dosage treatment of No Observed Adverse Effect Level (NOAEL).

1.3 Ruxolitinib

Ruxolitinib is a new drug approved by the United State Food and Drug Agency on 6 November 2011 under market name of Jakafi oral tablets from Incyte Corporation to treat intermediate and high-risk myelofibrosis which includes primary myelofibrosis, post-polycythaemia vera myelofibrosis and post-essential thrombocythemia myelofibrosis (FDA Report from https://www.accessdata.fda.gov).

Heine *et al.,* (2013) indicates that ruxolitinib proves to be effective in treating myelofibrosis with marked constitutional symptoms fatigue, night sweats, fever, weight loss, pruritus and symptoms of massive hepatosplenomegaly which sees its profound anti-inflammatory effects with spleen size decrease in patients. It also showed a reduction of cytokines. Verstovsek *et al.*, (2017) also observed the safety profile of ruxolitinib in long-term use on patients and concluded that the drug is effective to treat intermediate-2 and high-risk myelofibrosis.

Ruxolitinib is a small molecule with a molecular weight of 306.37gm/mol. It is orally administered to selectively inhibit JAK1 and JAK2 phosphorylation which subsequently would stop/reduce the hyperphosphorylation of STATs. It has been shown effective in treating myelofibrosis. According to Yi *et al.* (2015), ruxolitinib

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has approved myelofibrosis indications by improving myelofibrosis-related splenomegaly and other symptoms. Other than that, Shi *et al*. (2011) reported that oral dose of ruxolitinib was trialled in healthy volunteers in 2 double-blinded, randomised and placebo-controlled studies were showing proper metabolism and clearance with negligible excretion of doses of 5 to 200mg/kg. Ruxolitinib has 50% inhibitory concentration (IC50) *in vitro* of 3.3 nmol/L for JAK1 while it was 2.8 nmol/L for JAK2 (FDA Report from https://www.accessdata.fda.gov).

Having the ability to inhibit the proliferation of erythroid and myeloid progenitor cells in polycythaemia vera patients, it also reduces circulating inflammatory cytokines in murine models. Rapidly absorbed within 1 to 2 hour following oral administration, ruxolitinib can be ingested with or without a prior meal. It binds to albumin and be excreted via faeces thus making it a potentially safe drug candidate for general prepregnancy consumption and also throughout pregnancy.

The rationale of this study is to observe the potentiality of ruxolitinib to inhibit JAK-STAT pathway by suppressing gliogenesis thus enhancing neurogenesis in the brain of developing mouse foetus.

1.4 Problem Statement

Low initial neurones and lower neuronal density are found in the brain of Down syndrome child (Wisniewski 1990). It is related to neuronic-to-gliogenic shift during brain development, which sees the involvement of JAK-STAT pathway.

1.5 Research Hypothesis

Ruxolitinib supplementation suppresses JAKs phosphorylation and glial cells markers but enhances neuronal cells markers in the developing foetal mouse brain.

1.6 Expected Results

It is anticipated that Ruxolitinib treatment on a pregnant mouse serves as an introduction to a safe supplementation towards the pregnant mouse. Normal

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homeostatic range and morphology of blood profile, liver, kidney and spleen of the maternal mouse would substantiate no adverse effect after continuous consumption of the drug starting E7.5 until delivery which accounts for 14 days of pregnancy. It should also accord with a positive result on neuron-to-glia ratio enhancement in the pups' brain that this novel approach would support its application onto human prepregnancy supplementation that required further investigations.

1.7 Objectives

Ultimately, the objective of this study is to investigate the potential suppressive ability of Ruxolitinib on JAK-STAT signalling pathway in order to increase the neurogenic-to-gliogenic ratio in the foetal mouse brain.

The study has the following specific objectives:

i. To establish a safe dosage of Ruxolitinib supplement on pregnant mice sufficient to inhibit JAK-STAT signalling pathway during foetal development.

ii. To investigate the levels of neuronal and glial cells markers in the foetal mouse brain after Ruxolitinib supplementation.

1.8 Significance of Study

The benefits of this research are:

i. This study will give an insight into a potential hope to treat using Ruxolitinib on Down syndrome at an early embryogenesis stage to help alleviate the burden of parents and caregivers. A better initial number of functional neuron means a good head start. Also, this will bring hope onto patients to improve cognitive function, live longer and meaningful life.

ii. Economically, this will be multi-pronged devise into a cost-effective treatment of using the established drug to treat a genetic disorder that also reduces cost on caregiving on the 2017 total budget for Ministry of Health which accounts for RM 25 billion.

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He started working with the government of Malaysia as a Pegawai Tadbir dan Diplomatik in 2002 yet his passion for science has never faded. In 2015 he was granted a full scholarship study leave to do a Master of Science degree under the Jabatan Perkhidmatan Awam's Hadiah Latihan Persekutuan Cuti Belajar Bergaji Penuh under Dr. Chan Yoke Mun of Institut Gerontologi, UPM for a study on Alzheimer's disease and then was offered to join Dr. Michael Ling King Hwa's team in the field of neuroscience to work on novel approach of using 2011 USFDA approved myeloproliferative disease treatment drug to enhance neurogenesis in mouse model.

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