UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF CONDITIONAL WNT5A TRANSGENIC EMBRYONIC STEM CELL LINE AND ITS APPLICATION DURING NEURAL DIFFERENTIATION PROCESS

NURUL AFIQAH BINTI MOHAMED HISHAM

FPSK(M) 2017 55

ESTABLISHMENT OF CONDITIONAL WNT5A TRANSGENIC EMBRYONIC STEM CELL LINE AND ITS APPLICATION DURING NEURAL DIFFERENTIATION PROCESS

By

NURUL AFIQAH BINTI MOHAMED HISHAM

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

September 2017

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

ESTABLISHMENT OF CONDITIONAL WNT5A TRANSGENICEMBRYONIC STEM CELL LINE AND ITS APPLICATION DURING NEURAL DIFFERENTIATION PROCESS.

By

NURUL AFIQAH MOHAMED HISHAM

September 2017

Chairman: Norshariza Nordin, PhD
Faculty : Medicine and Health Sciences

One of the properties of embryonic stem (ES) cells is their differentiation potential including the capacity to form specific regional neural cells identity. However, limitation in getting homogenous neuronal specific subtype population from ES cells has resulted in the formation of teratoma upon transplantation, thus has hindered their use in clinical applications. Hence, understanding the key regulators during differentiation of ES cells is essential. Among the regulators, Wnt5a signalling molecule has been shown to play important role in neural differentiation process of mouse ES cells in a stage-dependent manner. Therefore, a system that allows for a tight regulation of Wnt5a expression in undifferentiated ES cells and also upon differentiation is indispensable in order to evaluate the stage-dependency effect of Wnt5a during the process. This study aims to generate and characterize Wnt5a transgenic cell line that carries the inducible Wnt5a transgene construct through a binary *Cre/loxP* system and to preliminarily evaluate its application in understanding the stage dependency effect of Wnt5a during the neural differentiation process. Two clones of Wnt5a transgenic line were successfully generated by transfecting the pCAG-floxed-*neo*pA-Wnt5a plasmid into a *Cre* expressing cell line, R26CT2S. Stable transfected cells were screened by dual antibiotic selections before and after exposure to 4'-hydroxytamoxifen (4'-OHT). The cell line was found to maintain its pluripotency. The expression of Wnt5a transgene was observed to be temporally controlled upon exposure to a non-detrimental dosage of 4'-OHT. High level of transgene expression was observed in clones induced with 4'-OHT both in ES cells and the embryoid bodies (EBs), clearly indicating the stability and inducibility of the Wnt5a construct. The generated inducible Wnt5a transgenic ES cell line was then applied to preliminarily understand the effects of Wnt5a activity at specific time points during neural differentiation process. The formation of multicellular aggregates, embryoid bodies (EBs) and the addition of retinoic acid in the presence of serum was chosen to differentiate ES cells into neural lineage. The expression of Wnt5a transgene was induced at three different time points: 1) early and 2) late stages of neural differentiation process and 3) constitutive expression (since the undifferentiated stage). The expression of selected specific neural markers for the formation of post-

mitotic, mature and dopaminergic (DA) neurons and astroglial cells was qualitatively and quantitatively analysed. The effects of stimulating Wnt5a signalling pathway at the specific time points were analysed at three different stages of the neural differentiation process; day 2, day 8 and day 16 post-plating neural culture. Interestingly without influence of any standard patterning factors, high and early detection of TH positive neuron, Class III β-tubulin and Map2 markers was observed when Wnt5a was induced at early stage of neural differentiation process. A dynamic expression pattern of the neural proteins generated, indicates the complex roles of Wnt5a during the process. This study, highlights the application of the conditional ES cell system in elucidating stage-dependency effect of Wnt5a during neural differentiation process and exposes the potential role of this molecule in generating ES cells-derived neural cells that are suitable for cell-based therapy for neurodegenerative diseases.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENUBUHAN KONDISIONAL WNT5A TRANSGENIK SEL STEM EMBRIONIK DAN APLIKASINYA SEMASA PROSES PEMBEZAAN NEURON.

Oleh

NURUL AFIQAH MOHAMED HISHAM

September 2017

Pengerusi : Norshariza Nordin, PhD
Fakulti : Perubatan dan Sains Kesihatan

Salah satu ciri unik sel stem embrionik (ES) adalah potensi pembezaannya termasuklah kemampannya membentuk identiti neuron yang spesifik. Namun begitu, terdapat batasan dalam mendapatkan subjenis populasi neuron yang sekata daripada sel stem embrionik yang mana menyebabkan pembentukan teratoma selepas implantasi lalu membataskan penggunaan sel stem embrionik dalam aplikasi klinikal. Oleh itu, pemahaman mengenai molekul yang mengawal pembezaan sel stem embrionik adalah sangat penting. Molekul isyarat Wnt5a telah dibuktikan memainkan peranan yang penting dalam proses pembezaan neuron daripada sel embrionik tikus, secara kesandaran peringkat. Oleh yang demikian, satu sistem yang dapat mengawal ketat regulasi ekspresi Wnt5a oleh sel stem embrionik sebelum dan selepas pembezaan neuron perlu diwujudkan bagi mengukur kesan kesandaran peringkat Wnt5a sewaktu proses tersebut. Kajian ini dijalankan bagi menghasilkan dan memperinci sel transgenik Wnt5a yang membawa konstruk transgen teraruh Wnt5a melalui sistem binari *Cre*/loxP serta mengkaji aplikasi sistem tersebut bagi memahami kesan kesandaran peringkat Wnt5a sewaktu proses pembezaan neuron. Dua klon sel transgenik Wnt5a telah berjaya dihasilkan melalui kaedah transfeksi plasmid pCAG-floxed-*neo*pA-Wnt5a ke dalam titisan sel yang mengekspreskan *Cre*, R26CT2S. Sel terinfeksi yang stabil disaring melalui seleksi berperingkat oleh dual antibiotik sebelum dan selepas pendedahan dengan 4'-hydroxytamoxifen (4'-OHT). Titisan sel tersebut didapati dapat mengekalkan ciri pluripotent sel. Expresi transgen Wnt5a didapati dikawal secara temporal selepas pendedahan kepada dos selamat 4-OHT. Ekspresi transgen yang tinggi turut dikesan pada klon yang diaruh dengan 4'-OHT dalam kedua-dua sel stem embrionik dan jasad embrio, menunjukkan kestabilan dan keboleharuhan konstruk Wnt5a tersebut. Titisan sel embrionik ini kemudiannya digunakan bagi mengkaji kesan aktiviti Wnt5a pada sela masa spesifik sewaktu proses pembezaan neuron. Kaedah, jasad embrio dan penambahan asid retinoik dengan kehadiran serum digunakan bagi membeza sel stem embrionik kepada nasab neural. Ekspresi transgen Wnt5a diaruh pada tiga masa berbeza: 1) awal 2) lewat proses pembezaan dan 3) ekpresi konstitutif (ekpresi Wnt5a sebelum pembezaan sel stem). Ekspresi penanda neural yang spesifik bagi neuron pasca mitotik, matang, dan dopaminergik serta sel astroglial dianalisa secara kualitatif dan kuantitatif. Kesan

stimulasi laluan penanda Wnt5a pada sela masa spesifik dianalisa pada 3 fasa berbeza dalam proses pembezaan neuron; hari ke 2, hari ke 8 dan hari ke16 pasca penyemaian kultur neural. Menariknya tanpa penambahan sebarang faktor pencorakan, neuron tirosin hydroxylase, kelas III β-tubulin dan Map2 positif dapat dikesan awal dan pada kadar yang tinggi, apabila Wnt5a diaruh di awal proses pembezaan neuron. Pola ekspresi penanda protein neural, menandakan peranan Wnt5a yang kompleks sewaktu proses tersebut. Kajian ini menunjukkan aplikasi sistem sel embrionik kondisional dalam menguraikan kesan kesandaran peringkat Wnt5a seterusnya membuktikan potensi molekul ini dalam usaha menghasilkan sel neuron daripada sel stem embrionik. Sel neuron daripada sel stem ini pula sesuai digunakan dalam terapi berasaskan sel terutama dalam merawat penyakit neurodegeneratif.

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious, The Most Merciful. All thanks and praises to Allah, The Almighty for granting me life, good health and strength. Without His Will, I would not be able to complete my thesis successfully. This thesis represents the result of many experiences I have encountered at Faculty of Medicine and Health Sciences, Universiti Putra Malaysia from dozens of remarkable individuals. I would therefore like to offer my sincere gratitude to all people that were involved in my research, in a direct or indirect way, who enabled me to obtain quality data needed for my thesis research. May Allah bless all of you till Jannah.

First and foremost, I would like to warmly thank and appreciate my parents, Mohamed Hisham Ibrahim, ayah and Norah binti Md Noor, Ibu for the love, care and spiritual support in all of my life. Thank you for being patience with me. I would also like to thank my sister Khairul Barieyah, along and brothers, Amar and Amirul for they have provided assistance in numerous ways. May Allah give you all the best in return.

I would like to take this opportunity to express my deepest gratitude to my supervisor, Dr. Norshariza Nordin, for all her help, guidance, encouragement and advice throughout my project. Thank you Dr for being such a supportive supervisor, mother and friend to me. Thank you for being patience to hold my hands and guide me through this long and difficult journey. Deep down inside, I really wish your happiness in dunya and the day after! I would also like to thank my co-supervisor Dr. Michael Ling King Hwa for his useful help and advice throughout my entire studies. I'm also grateful to all the lecturers and staff of Stem Cell and Research Laboratory and also Medical Genetics Laboratory for all the help and support given throughout my time here. Of course appreciation goes to Kak Hazlen, Kak Salimah, and Kak Puspa from Medical Genetics Laboratory (MGL) for their assistance and hard work in keeping the lab well maintained.

Not forgetting all my colleagues and friends throughout my journey here. I would express my heartfelt gratitude towards my groupmates, Adila, Nur 'Izzati, Atiqah, Nurusaadah, Mun Fun, Khair and Carol. I would also like to thank all my beloved friends that have accompanied me through an eye-opening journey here especially, Marlini, Haslinda, Omar and Elson, Nadirah, Nj, Sulei, Nurul Afifah Aziz and Asween Sani, sulei My time here would not have been such colourful without any of you.
May Allah grant us all success in the world and in the hereafter, âmîn.

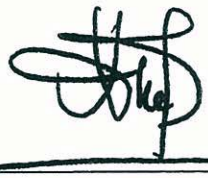
I certify that a Thesis Examination Committee has met on 7 September 2017 to conduct the final examination of Nurul Afiqah binti Mohamed Hisham on her thesis entitled "Establishment of Conditional Wnt5a Transgenic Embryonic Stem Cell Line and its Application During Neural Differentiation Process" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Syahrilnizam bin Abdullah, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Mohamad Aris bin Mohd Moklas, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Azlina Ahmad Annuar, PhD
Senior Lecturer
University of Malaya
Malaysia
(External Examiner)



NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 October 2017

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Norshariza Nordin, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Ling King-Hwa, PhD
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia.
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No.: Nurul Afiqah Binti Mohamed Hisham, GS38283

Declaration by Members of Supervisory Committee

This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

Signature: _____
Name of Chairman of
Supervisory Committee: Dr. Norshariza Nordin

Signature: _____
Name of Member of
Supervisory Committee: Dr. Ling King-Hwa

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

4'-OHT	4'-hydroxytamoxifen
APC	Adenomatous polyposis coli
ATRA	All trans retinoic acid
BSA	Bovine serum albumin
BMP	Bone mrphogenetic protein
CamKII	Ca^{2+} calmodulin-dependent protein kinase II
CE	Constitutively expressed
G418	Neomycin/geniticin
CK1	Casein kinase 1
CNS	Central nervous system
CRD	Cysteine-rich domain
DPC	Day-post coitum
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DVL	Dishevelled
ER	Estrogen receptor
ES	Embryonic stem
EBS	Embryoid bodies
FGF	Fibroblast growth factor
FBS	Fibroblast growth factor
FZ	Frizzled
GSK3	Glycogen synthase kinase 3
GFAP	Glial fibrillary acidic protein
ICM	Inner cell mass
LIF	Leukimia inhibitor factor
LEF	Lymphoid enhancer-binding factor
LRP	Low density lipoprotein receptor-related proteins
MAP2	Microtubule associated protein-2
Neo	Neomycin resistance gene
NFAT	Nuclear factor activated T-cells
NPC	Neural precursor cells
Pac	Puromycin resistance gene
PCR	Polymerase chain reaction
PLC	PhospHolipase-Cells
RE	Restriction enzyme
ROR	Receptor tyrosine kinase-like orphan receptor
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real-time quantitative PCR
RYK	Receptor-like tyrosine kinase
R26	Rosa 26

RXR	Retinoic X receptors
sFRPs	Secreted frizzled-related proteins
SHH	Sonic hedgehog
UTR	Untranslated region
VZ	Ventricular zone
mg/dL	Milligrams per deciliter
U/L	Units per litre
pg/mL	Picograms per milliliter
rpm	Revolutions per minute
mM	Millimolar
µM	Micromolar
µl	Microliter
µg/ml	Micrograms per milliliter
ng/µl	Nanograms per microliter
mmol/L	Millimole per liter
nmol/L	Nanomole per liter
V	Volt
s	Second
min	Minute
[ ]	Concentration
TAE	Tris Acetate-EDTA
$MgCl_2$	Magnesium chloride
bp	Base pair
kcal/mol	Kilocalorie per mole
PCR	Polymerase chain reaction
dNTPs	Deoxyribonucleotides
1x	One time
5x	Five times
ml	Milliliter
g	Grams
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic Acid
mRNA	Messenger RNA
3D	Three dimensional

CHAPTER 1

INTRODUCTION

The embryonic stem cells were first isolated by Evans and Kaufman in 1981. This cell was isolated from the inner cell mass of the mouse blastocyst around embryonic day (E) 3.5- 4.5. Embryonic stem (ES) cells acquire two main characteristics; the ability to maintain self-renewal capacity upon prolonged culture under appropriate condition and also the capability to differentiate into the three primary germ layers-derived cells *in vitro*. These two characteristics have made ES cells as a potential tool in understanding various processes during neural development, drugs discovery and most importantly, in regenerative medicine targeting particularly degenerative disease. In neurodegenerative diseases such as Parkinson's disease (PD), L-Dopa is a drug that is commonly prescribed for Parkinson's patient. Despite its efficacy in improving symptoms of Parkinsonian patients, this drug particularly works but with neuropsychiatric side effects (Morizane et al., 2008). Previous study on clinical trials of embryonic ventral mesencephalic tissue transplantation demonstrated symptomatic recovery and the grafted cells showed extensive re-innervation into the host tissue (Annet et al., 1994). However ethical concerns have limited embryonic ventral mesencephalic tissue supply. Alternatively, generation of cells or tissues from unlimited supply would be an attractive solution. The capacity of ES cell to generate neurons in culture to restore the damaged neurons, may serve as a potential alternative source of transplantable neurons for neurodegenerative diseases.

Currently, a number of group have established ES cell-derived functional defined neurons as a model for neurodegenerative diseases such as Alzheimer's disease and spinal cord injury (Ying et al,. 2003; Abranches et al,. 2009; Zhu et al,. 2016). One of the approaches in ES cell-based therapies is to generate highly homogenous population of neurons in culture and transplant them into the brain region to replace the neuronal loss. Previous study showed neurons derived from ES cells were successfully survived and established connections with the host cells (Andersson et al., 2013; Steinbeck et al., 2012), confirming the potential of ES cells as the source of cells for transplantation. However the current results were too preliminary to be taken into clinical practice. Among the major issues in ES cell-based therapies are the low survival rate of transplanted neurons and poor availability of homogeneous pure neurons in culture which will cause teratoma formation upon transplantation in a host. These are the main hindrance for their usage in clinical settings (Kooreman & Wu, 2010). Therefore, proper understanding of the underlying mechanisms that govern neural differentiation process is necessary in order to generate highly homogenous population of defined neuronal subtype.

There are many key regulators governing the neurogenesis process during development of the central nervous system (CNS). These factors which are required in neural patterning are attractive candidates in directing ES cells towards specific neural lineages. Wnt is among the members of related proteins that plays a critical role in the CNS development, besides sonic hedgehog (Shh) (Balaskas et al., 2012), bone

morphogenetic protein (BMP) (Ille et al., 2007) and retinoic acid (RA) (Wobus et al., 1997). To date 19 members of Wnt have been discovered in mammals, which are grouped into canonical (β-catenin pathway) and non-canonical pathways (β-catenin independent pathway). Wnt signalling has been shown to be involved in regulating the stem cell proliferation, anterior and posterior patterning of the embryo and also in the developmental processes of the CNS (Liu et al., 2008; Wan et al., 2014). Several *in vivo* and *in vitro* analyses showed the expression of Wnt signalling proteins are stage and regional dependent (Lange et al., 2006). The activity of Wnt is highly dependent on the type and developmental stage of the target cell, the same Wnt might regulate multiple roles at different cell context (Kunke et al., 2009).

Wnt5a belongs to Wnt signalling molecule which is categorized under the β-catenin independent pathway. The binding of Wnt5a on transmembrane receptor Frizzled (Fz) and co-receptor will generally transduce Wnt/Calcium and Wnt/Planar polarity pathways. Wnt5a was found to regulate body segmentation and polarity during embryonic stage (Bodmer et al., 2009; Yamaguchi et al., 1999). In addition, Wnt5a was also found in regulating the convergent extension process of *Xenopus* embryo (Sato et al., 2009). Further, the role of Wnt5a was discovered in axon morphogenesis (Blakely et al., 2013; Clark et al., 2014) and also synaptogenesis (Bodmer et al., 2009; Davis et al., 2008). In addition, stimulation of Wnt5a was found to promote differentiation of neural precursor cells (NPC), rather than maintaining the NPC population, showing that Wnt5a promotes cell specification of NPC (Nordin et al., 2008; Yu et al., 2006). An *in vivo* study conducted revealed deletion of Wnt5a in E10.5 mouse has increased progenitor proliferation in the midbrain floor plate (Andersson et al., 2008). These studies provided prove of the important roles of Wnt5a signalling in segmentation and also in neurogenesis.

To date limited studies have been carried out to understand the roles of Wnt5a during neural differentiation process of mouse ES cells. In an effort to investigate the efficiency of neural differentiation process of pluripotent cells, Kwon and colleagues (2014) have conducted microarray analysis. The finding showed, Wnt5a is one of the major regulatory genes that was upregulated during neural differentiation process of induced pluripotent stem cells and embryonic stem cells. Previous study has shown treatment of Wnt5a and Fgf2 at later stage of ES cell promoted the neural differentiation process *in vitro* which then upon transplanted into animal model enhanced the survival of functional neuron *in vivo* (Sanchez-Pernaute et al., 2008). This observation clearly suggest the stage dependency of Wnt5a in regulating neurogenesis. Supporting the finding, a study conducted by Nordin et al (2008) on neural differentiation from mouse ES cells by 4-/4+ protocol, has shown a dynamic expression of Wnt5a transcript where its activity is believed to be stage dependent. The stage dependency effect of Wnt5a was further tested by Anderson et al (2013) by sequential stimulation of Wnt3a and Wnt5a where a significant increase of tyrosine hydroxylase (TH) positive cells was observed, indicating the stage-specific activity of Wnt is required to control different stages of midbrain DA neurons. All these findings suggest the involvement of Wnt5a in regulating neural differentiation *in vivo* and *in vitro* in stage dependent manner.

The Wnt5a activity is complex and its expression during neural differentiation process has been poorly understood. The used of purified Wnt5a protein to observe the effect of Wnt5a during the differentiation process has been reported. However, direct protein treatment might hinder the comprehensive quantitative biochemical analysis due to dilution of its concentration by the medium and other external factors [review in (Van Amerongen & Nusse 2009)]. In order to evaluate the stage-dependency effect of Wnt5a during the process, a system that allows us to tightly regulate its expression not only in undifferentiated ES cells but also upon differentiation in a closed environment, within the cells is highly needed.

In this study *Cre*-loxP based ES cell expression system is utilized to establish inducible Wnt5a transgenic ES cell line. The transgenic line would be useful in stimulating Wnt5a signalling at specific time points during neural differentiation of ES cells. Hence, the system is allowing the evaluation of the stage-dependant effect of Wnt5a during the process to be carried out. The key questions of this study are; 1) Can stable and inducible Wnt5a transgenic cell line be established? 2) Can Wnt5a transgenic cell line be used in assessing the stage dependency effect of Wnt5a during neural differentiation process at different time point?

Hypothesis

A stable and inducible Wnt5a transgenic ES cell line was successfully established, and it has high potential to unravel the stage dependency effect of Wnt5a during neural differentiation process of mouse ES cell.

General objectives

To establish inducible Wnt5a transgenic line and to preliminarily unravel the stage-dependent role of Wnt5a during neural differentiation process of mouse ES cells.

Specific aims:

1) To validate the inducible Wnt5a construct plasmid
2) To establish and characterize conditional Wnt5a transgenic mouse ES cell line.
3) To preliminarily analyse the effect of inducing Wnt5a transgene at three different time points during neural differentiation process on the expression of selected neural protein markers for post-mitotic, mature and dopaminergic neurons, and astroglial cell.

REFERENCES

Bain, G., Kitchens, D., Yao, M., Huettner, E,J., Gottlieb, I,D. Embryonic Stem Cells Express Neuronal Properties In Vitro. (1995). *Developmental Biology*, 168, 342-357.

Abeliovich, A., & Hammond, R. (2007). Midbrain dopamine neuron differentiation: Factors and fates. *Developmental Biology,* 304(2), 447-454. http://doi.org/10.1016/j.ydbio.2007.01.032

Abranches, E., Silva, M., Pradier, L., Schulz, H., Hummel, O., Henrique, D., & Bekman, E. (2009). Neural differentiation of embryonic stem cells in vitro: a road map to neurogenesis in the embryo. *PLoS One*, *4*(7), e6286. http://doi.org/10.1371/journal.pone.0006286

Alexopoulou, A. N., Couchman, J. R., & Whiteford, J. R. (2008). The CMV early enhancer / chicken β actin (CAG) promoter can be used to drive transgene expression during the differentiation of murine embryonic stem cells into vascular progenitors. *BMC Cell Biology*, 9 (2), 1–11. http://doi.org/10.1186/1471-2121-9-2

Anastassiadis, K., Fu, J., Patsch, C., Hu, S., Weidlich, S., Duerschke, K., Stewart, A. F. (2009). Dre recombinase, like Cre, is a highly efficient site-specific recombinase in E. coli, mammalian cells and mice. *Disease Models & Mechanisms*, *2*(9-10), 508–515. http://doi.org/10.1242/dmm.003087

Andersson, E. R., Prakash, N., Cajanek, L., Minina, E., Bryja, V., Bryjova, L., Arenas, E. (2008). Wnt5a regulates ventral midbrain morphogenesis and the development of A9-A10 dopaminergic cells in vivo. *PLoS ONE*, *3*(10). http://doi.org/10.1371/journal.pone.0003517

Andersson, E. R., Saltó, C., Villaescusa, J. C., Cajanek, L., Yang, S., & Bryjova, L. (2013). Wnt5a cooperates with canonical Wnts to generate midbrain dopaminergic neurons in vivo and in stem cells, *110*(7). http://doi.org/10.1073/pnas.1208524110

Andre, P., Song, H., Kim, W., Kispert, A., & Yang, Y. (2015). Wnt5a and Wnt11 regulate mammalian anterior-posterior axis elongation. *Development (Cambridge, England)*, dev.119065. http://doi.org/10.1242/dev.119065

Andre, P., Wang, Q., Wang, N., Gao, B., Schilit, A., Halford, M. M., Yang, Y. (2012). The Wnt coreceptor Ryk regulates Wnt/planar cell polarity by modulating the degradation of the core planar cell polarity component Vangl2. *Journal of Biological Chemistry*, *287*(53), 44518–44525. http://doi.org/10.1074/jbc.M112.414441

Arenas, E. (2014). Wnt signaling in midbrain dopaminergic neuron development and regenerative medicine for Parkinson ' s disease. *Journal of Molecular Cell Biology*, 6(1), 42–53. http: // doi.org/10.1093/jmcb/mju001

Aubert, J., Dunstan, H., Chambers, I., & Smith, A. (2002). Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. *Nature Biotechnology*, *20*(12), 1240–1245. http://doi.org/10.1038/nbt763

Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., & Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes and Development*, *17*(1), 126–140. http://doi.org/10.1101/gad.224503

Baizabal, J. M., & Covarrubias, L. (2009). The embryonic midbrain directs neuronal specification of embryonic stem cells at early stages of differentiation. *Developmental Biology*, *325*(1), 49–59. http://doi.org/10.1016/j.ydbio.2008.09.024

Balaskas, N., Ribeiro, A., Panovska, J., Dessaud, E., Sasai, N., Page, K. M., Ribes, V. (2012). Gene regulatory logic for reading the sonic hedgehog signaling gradient in the vertebrate neural tube. *Cell*, *148*(1-2), 273–284. http://doi.org/10.1016/j.cell.2011.10.047

Balmer, J. E. (2002). Gene expression regulation by retinoic acid. *The Journal of Lipid Research*, *43*(11), 1773–1808. http://doi.org/10.1194/jlr.R100015-JLR200

Bielen, H., & Houart, C. (2014). The Wnt cries many: Wnt regulation of neurogenesis through tissue patterning, proliferation, and asymmetric cell division. *Developmental Neurobiology*, *74*(8), 772–780. http://doi.org/10.1002/dneu.22168

Blainey, P., Krzywinski, M., & Altman, N. (2014). Points of Significance: Replication. *Nature Methods*, *11*(9), 879–880. http://doi.org/10.1038/nmeth.3091

Blakely, B. D., Bye, C. R., Fernando, C. V, Horne, M. K., Macheda, M. L., Stacker, S. A., Parish, C. L. (2011). Wnt5a Regulates Midbrain Dopaminergic Axon Growth and Guidance, *6*(3). http://doi.org/10.1371/journal.pone.0018373

Blakely, B. D., Bye, C. R., Fernando, C. V, Prasad, A. a, Pasterkamp, R. J., Macheda, M. L., Parish, C. L. (2013). Ryk, a receptor regulating Wnt5a-mediated neurogenesis and axon morphogenesis of ventral midbrain dopaminergic neurons. *Stem Cells and Development*, *22*(15), 2132–44. http://doi.org/10.1089/scd.2013.0066

Bodmer, D., Levine-Wilkinson, S., Richmond, A., Hirsh, S., & Kuruvilla, R. (2009). Wnt5a mediates nerve growth factor-dependent axonal branching and growth in developing sympathetic neurons. *The Journal of Neuroscience : The Official*

Journal of the Society for Neuroscience, *29*(23), 7569–7581. http://doi.org/10.1523/JNEUROSCI.1445-09.2009

Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell*, *122*(6), 947–956. http://doi.org/10.1016/j.cell.2005.08.020

Cai, L., Ye, Z., Zhou, B. Y., Mali, P., Zhou, C., & Cheng, L. (2007). Promoting human embryonic stem cell renewal or differentiation by modulating Wnt signal and culture conditions. *Cell Research*, *17*(1), 62–72. http://doi.org/10.1038/sj.cr.7310138

Castelo-Branco, G., Sousa, K. M., Bryja, V., Pinto, L., Wagner, J., & Arenas, E. (2006). Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion. *Molecular and Cellular Neuroscience*, *31*(2), 251–262. http://doi.org/10.1016/j.mcn.2005.09.014

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature*, *450*(7173), 1230–4. http://doi.org/10.1038/nature06403

Chatterjee, P., Cheung, Y., & Liew, C. (2011). Transfecting and Nucleofecting Human Induced Pluripotent Stem Cells, (October), 10–13. http://doi.org/10.3791/3110

Chenn, A., & Walsh, C. A. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science (New York, N.Y.)*, *297*(5580), 365–9. http://doi.org/10.1126/science.1074192

Cho, M. S., Lee, Y.-E., Kim, J. Y., Chung, S., Cho, Y. H., Kim, D.-S., Kim, D.-W. (2008). Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. *Proceedings of the National Academy of Sciences*, *105*(9), 3392–7. http://doi.org/10.1073/pnas.0712359105

Ciani, L., & Salinas, P. C. (2005). WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nature Reviews. Neuroscience*, *6*(5), 351–362. http://doi.org/10.1038/nrn1665

Clark, C. E. J., Richards, L. J., Stacker, S. a, & Cooper, H. M. (2014). Wnt5a induces Ryk-dependent and -independent effects on callosal axon and dendrite growth. *Growth Factors*, *32*(1), 11–17. http://doi.org/10.3109/08977194.2013.875544

Clevers, H., & Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell*, 149(6), 1192-205. http://doi.org/10.1016/j.cell.2012.05.012

Coucouvanis, E., & Martin, G. R. (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. *Development (Cambridge, England)*, *126*(3), 535–546.

Davis, E. K., Zou, Y., & Ghosh, A. (2008). Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. *Neural Development*, *3*, 32. http://doi.org/10.1186/1749-8104-3-32

De, A. (2011). Wnt / Ca 2 1 signaling pathway : a brief overview The Non-canonical Wnt Signaling Cascade, *43*(10), 745–756. http://doi.org/10.1093/abbs/gmr079

De Los Angeles, A., Ferrari, F., Xi, R., Fujiwara, Y., Benvenisty, N., Deng, H., Daley, G. Q. (2015). Hallmarks of pluripotency. *Nature*, *525*(7570), 469–78. http://doi.org/10.1038/nature15515

Deng, C. (2012). The Use of Cre – loxP Technology and Inducible Systems to Generate Mouse Models of Cancer, 17–37. http://doi.org/10.1007/978-0-387-69805-2

Dent, E. W., & Baas, P. W. (2014). Microtubules in neurons as information carriers. *Journal of Neurochemistry*. http://doi.org/10.1111/jnc.12621

Feil, R., Wagner, J., Metzger, D., & Chambon, P. (1997). Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochemical and Biophysical Research Communications*, *237*(3), 752–757. http://doi.org/10.1006/bbrc.1997.7124

Filipczyk, A., Marr, C., Hastreiter, S., Feigelman, J., Schwarzfischer, M., Hoppe, P. S., Schroeder, T. (2015). Network plasticity of pluripotency transcription factors in embryonic stem cells. *Nature Cell Biology*, *17*(10), 1235–1246. http://doi.org/10.1038/ncb3237

Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W.-Y., DuMouchel, W., Kao, R., Fahn, S. (2001). Transplantation of Embryonic Dopamine Neurons for Severe Parkinson's Disease. *New England Journal of Medicine*, *344*(10), 710–719. http://doi.org/10.1056/NEJM200103083441002

Fuhrmann, G., Chung, A. C. K., Jackson, K. J., Hummelke, G., Baniahmad, A., Sutter, J., Cooney, A. J. (2001). Mouse Germline Restriction of Oct4 Expression by Germ Cell Nuclear Factor. *Developmental Cell*, *1*(3), 377–387. http://doi.org/10.1016/S1534-5807(01)00038-7

Gao, B., Song, H., Bishop, K., Elliot, G., Garrett, L., English, M. A., Yang, Y. (2011). Wnt Signaling Gradients Establish Planar Cell Polarity by Inducing Vangl2 Phosphorylation through Ror2. *Developmental Cell*, *20*(2), 163–176. http://doi.org/10.1016/j.devcel.2011.01.001

Gao, C., & Chen, Y. G. (2010). Dishevelled: The hub of Wnt signaling. *Cellular Signalling*. http://doi.org/10.1016/j.cellsig.2009.11.021

Gaspard, N., Bouschet, T., Herpoel, A., Naeije, G., van den Ameele, J., & Vanderhaeghen, P. (2009). Generation of cortical neurons from mouse embryonic

stem cells. *Nature Protocols*, *4*(10), 1454–1463. http://doi.org/10.1038/nprot.2009.157

Gierut, J. J., Jacks, T. E., & Haigis, K. M. (2014). Strategies to achieve conditional gene mutation in mice. *Cold Spring Harbor Protocols*, *2014*(4), 339–349. http://doi.org/10.1101/pdb.top069807

Gofflot, F., Hall, M., & Morriss-Kay, G. M. (1997). Genetic patterning of the developing mouse tail at the time of posterior neuropore closure. *Developmental Dynamics*, *210*(4), 431–445. http://doi.org/10.1002/(SICI)1097-0177(199712)210:4<431::AID-AJA7>3.0.CO;2-H

Gordon, M. D., & Nusse, R. (2006). Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *Journal of Biological Chemistry*, 281(32), 22429-33. http://doi.org/10.1074/jbc.R600015200

Grainge, I., & Sherratt, D. J. (1999). Xer site-specific recombination. DNA strand rejoining by recombinase XerC. *Journal of Biological Chemistry*, *274*(10), 6763–6769. http://doi.org/10.1074/jbc.274.10.6763

Grandel, H., Lun, K., Rauch, G.-J., Rhinn, M., Piotrowski, T., Houart, C., Brand, M. (2002). Retinoic acid signalling in the zebrafish embryo is necessary during pre-segmentation stages to pattern the anterior-posterior axis of the CNS and to induce a pectoral fin bud. *Development*, *129*(12), 2851–2865. Retrieved from http://dev.biologists.org/content/129/12/2851.long

Haegele, L., Ingold, B., Naumann, H., Tabatabai, G., Ledermann, B., & Brandner, S. (2003). Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression. *Molecular and Cellular Neuroscience*, *24*(3), 696–708. http://doi.org/10.1016/S1044-7431(03)00232-X

Halleskog, C., Dijksterhuis, J. P., Kilander, M. B. C., Becerril-Ortega, J., Villaescusa, J. C., Lindgren, E., Schulte, G. (2012). Heterotrimeric G protein-dependent WNT-5A signaling to ERK1/2 mediates distinct aspects of microglia proinflammatory transformation. *Journal of Neuroinflammation*, *9*(1), 111. http://doi.org/10.1186/1742-2094-9-111

Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N., & Gotoh, Y. (2004). The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development*, *131*(12), 2791–2801. http://doi.org/10.1242/dev.01165

Ille, F., Atanasoski, S., Falk, S., Ittner, L. M., Märki, D., Büchmann-Møller, S., Sommer, L. (2007). Wnt/BMP signal integration regulates the balance between proliferation and differentiation of neuroepithelial cells in the dorsal spinal cord. *Developmental Biology*, *304*(1), 394–408. http://doi.org/10.1016/j.ydbio.2006.12.045

Indra, A. K., Warot, X., Brocard, J., Bornert, J. M., Xiao, J. H., Chambon, P., & Metzger, D. (1999). Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: Comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Research*, *27*(22), 4324–4327. http://doi.org/10.1093/nar/27.22.4324

Jang, S., Park, J.-S., & Jeong, H.-S. (2015). Neural Differentiation of Human Adipose Tissue-Derived Stem Cells Involves Activation of the Wnt5a/JNK Signalling. *Stem Cells International*, *2015*(Dvl), 1–7. http://doi.org/10.1155/2015/178618

Jha, B. S., Rao, M., & Malik, N. (2015). Motor Neuron Differentiation from Pluripotent Stem Cells and Other Intermediate Proliferative Precursors that can be Discriminated by Lineage Specific Reporters. *Stem Cell Reviews and Reports*, *11*(1), 194–204. http://doi.org/10.1007/s12015-014-9541-0

Junghans, D., Hack, I., Frotscher, M., Taylor, V., & Kemler, R. (2005). β-catenin-mediated cell-adhesion is vital for embryonic forebrain development. In *Developmental Dynamics* (Vol. 233, pp. 528–539). http://doi.org/10.1002/dvdy.20365

Kaufman, W. L., Kocman, I., Agrawal, V., Rahn, H., Besser, D., Gossen, M., & Delbru, M. (2008). Homogeneity and persistence of transgene expression by omitting antibiotic selection in cell line isolation, *36*(17). http://doi.org/10.1093/nar/gkn508

Kawano, Y., & Kypta, R. (2003). Secreted antagonists of the Wnt signalling pathway. *Journal of Cell Science*, *116*(Pt 13), 2627–2634. http://doi.org/10.1242/jcs.00623

Kawasaki, H., Mizuseki, K., Nishikawa, S., Kaneko, S., Kuwana, Y., Nakanishi, S., Sasai, Y. (2000). Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron*, *28*(1), 31–40. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11086981

Keeble, T. R., Halford, M. M., Seaman, C., Kee, N., Macheda, M., Anderson, R. B., Cooper, H. M. (2006). The Wnt Receptor Ryk Is Required for Wnt5a-Mediated Axon Guidance on the Contralateral Side of the Corpus Callosum, *26*(21), 5840–5848. http://doi.org/10.1523/JNEUROSCI.1175-06.2006

Keller, G. (2005). Embryonic stem cell differentiation : emergence of a new era in biology and medicine. *Genes and Development,* 19(10), 1129–1155. http://doi.org/10.1101/gad.1303605.)

Kibar, Z., Torban, E., McDearmid, J. R., Reynolds, A., Berghout, J., Mathieu, M., Gros, P. (2007). Mutations in VANGL1 associated with neural-tube defects. *The New England Journal of Medicine*, *356*(14), 1432–7. http://doi.org/10.1056/NEJMoa060651

Kikuchi, A., Yamamoto, H., Sato, A., & Matsumoto, S. (2011). New Insights into the Mechanism of Wnt Signaling Pathway Activation. *International Review of Cell and Molecular Biology*, *291*, 21–71. http://doi.org/10.1016/B978-0-12-386035-4.00002-1

Koch, P., Opitz, T., Steinbeck, J. A., Ladewig, J., & Brüstle, O. (2009). A rosette-type, self-renewing human ES cell-derived neural stem cell with potential for in vitro instruction and synaptic integration. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(9), 3225–30. http://doi.org/10.1073/pnas.0808387106

Kooreman, N. G., & Wu, J. C. (2010). Tumorigenicity of pluripotent stem cells: biological insights from molecular imaging. *Journal of the Royal Society, Interface / the Royal Society*, *7 Suppl 6*, S753–63. http://doi.org/10.1098/rsif.2010.0353.focus

Kopke, K., Hoff, B., & Kuck, U. (2010). Application of the saccharomyces cerevisiae FLP/FRT recombination system in filamentous fungi for marker recycling and construction of knockout strains devoid of heterologous genes. *Applied and Environmental Microbiology*, *76*(14), 4664–4674. http://doi.org/10.1128/AEM.00670-10

Kunke, D., Bryja, V., Mygland, L., Arenas, E., & Krauss, S. (2009). Inhibition of canonical Wnt signaling promotes gliogenesis in P0-NSCs. *Biochemical and Biophysical Research Communications*, *386*(4), 628–633. http://doi.org/10.1016/j.bbrc.2009.06.084

Kuroda, T., Tada, M., Kubota, H., Kimura, H., Hatano, S., Suemori, H., Tada, T. (2005). Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Molecular and Cellular Biology*, *25*(6), 2475–85. http://doi.org/10.1128/MCB.25.6.2475-2485.2005

Kurosawa, H. (2007). Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *Journal of Bioscience and Bioengineering*, *103*(5), 389–98. http://doi.org/10.1263/jbb.103.389

Kwon, Y.-W., Chung, Y.-J., Kim, J., Lee, H.-J., Park, J., Roh, T.-Y., Kim, H.-S. (2014). Comparative Study of Efficacy of Dopaminergic Neuron Differentiation between Embryonic Stem Cell and Protein-Based Induced Pluripotent Stem Cell. *PloS One*, *9*(1), e85736. http://doi.org/10.1371/journal.pone.0085736

Lange, C., Mix, E., Rateitschak, K., & Rolfs, A. (2006). Wnt signal pathways and neural stem cell differentiation. *Neurodegenerative Diseases*, *3*(1-2), 76–86. http://doi.org/10.1159/000092097

Leite, F., Lima, M., Marino, F., Cosentino, M., & Ribeiro, L. (2016). Dopaminergic Receptors and Tyrosine Hydroxylase Expression in Peripheral Blood Mononuclear Cells: A Distinct Pattern in Central Obesity. *Plos One*, *11*(1), e0147483. http://doi.org/10.1371/journal.pone.0147483

Li, L., Bennett, S. A. L., & Wang, L. (2012). Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. *Cell Adhesion & Migration*, *6*(1), 59–73. http://doi.org/10.4161/cam.19583

Li, L., Hutchins, B. I., & Kalil, K. (2009). Wnt5a Induces Simultaneous Cortical Axon Outgrowth and Repulsive Axon Guidance through Distinct Signaling Mechanisms. *The Journal of Neuroscience, 29*(18), 5873–5883. http://doi.org/10.1523/JNEUROSCI.0183-09.2009

Li, X., Guan, Y., Chen, Y., Zhang, C., Shi, C., Zhou, F., Wang, X. (2013). Expression of Wnt5a and its receptor Fzd2 is changed in the spinal cord of adult amyotrophic lateral sclerosis transgenic mice. *International Journal of Clinical and Experimental Pathology*, *6*(7), 1245–1260

Liew, C, G., Draper, J. S., Walsh, J., Moore, H., & Andrews, Andrews, P.W. (2007). Transient and Stable Transgene Expression in Human Embryonic Stem Cell. *Stem Cells* 25(6), 1521–1528. http://doi.org/10.1634/stemcells.2006-0634

Liu, Y., Wang, X., Lu, C.-C., Kerman, R., Steward, O., Xu, X.-M., & Zou, Y. (2008). Repulsive Wnt signaling inhibits axon regeneration after CNS injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(33), 8376–8382. http://doi.org/10.1523/JNEUROSCI.1939-08.2008

Liyang, G., Abdullah, S., Rosli, R., & Nordin, N. (2014). Neural commitment of embryonic stem cells through the formation of embryoid bodies (EBs). *Malaysian Journal of Medical Sciences, 21(5), 8-16.* https://www.ncbi.nlm.nih.gov/pubmed/25977628

Loebel, D. A., Watson, C. M., De Young, R. A., & Tam, P. P. (2003). Lineage choice and differentiation in mouse embryos and embryonic stem cells. *Developmental Biology*, *264*(1), 1–14. http://doi.org/10.1016/S0012-1606(03)00390-7

Loh, Y.H., Wu, Q., Chew, J.-L., Vega, V. B., Zhang, W., Chen, X., Ng, H.-H. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nature Genetics*, *38*(4), 431–440. http://doi.org/10.1038/ng1760

Louvi, A., & Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nature Reviews. Neuroscience*, *7*(2), 93–102. http://doi.org/10.1038/nrn1847

Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., & Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO Journal*, *18*(15), 4261–4269. http://doi.org/10.1093/emboj/18.15.4261

Megason, S. G., & McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development (Cambridge, England)*, *129*(9), 2087–2098.

Michaelidis, T. M., & Lie, D. C. (2008). Wnt signaling and neural stem cells: Caught in the Wnt web. *Cell and Tissue Research*, *331*(1), 193–210. http://doi.org/10.1007/s00441-007-0476-5

Mikels, A. J., & Nusse, R. (2006). Purified Wnt5a protein activates or inhibits β-catenin-TCF signaling depending on receptor context. *PLoS Biology*, *4*(4), 570–582. http://doi.org/10.1371/journal.pbio.0040115

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Yamanaka, S. (2003). The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, *113*(5), 631–642. http://doi.org/10.1016/S0092-8674(03)00393-3

Mun-Fun, H., Ferdaos, N., Hamzah, S. N., Ridzuan, N., Hisham, N. A., Abdullah, S., Nordin, N. (2015). Rat full term amniotic fluid harbors highly potent stem cells. *Research in Veterinary Science*, *102*, 89–99. http://doi.org/10.1016/j.rvsc.2015.07.010

Nagy, A. (2000). Cre recombinase: The universal reagent for genome tailoring. *Genesis*, *26*(2), 99–109. http://doi.org/10.1002/(SICI)1526-968X(200002)26:2<99::AID-GENE1>3.0.CO;2-B

Napoli, J. L. (1999). Interactions of retinoid binding proteins and enzymes in retinoid metabolism. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. http://doi.org/10.1016/S1388-1981(99)00117-1

Nishikawa, S. I., Jakt, L. M., & Era, T. (2007). Embryonic stem-cell culture as a tool for developmental cell biology. *Nature reviews Molecular cell biology*, *8*(6). http://doi: 10.1038/nrm2189

Niwa, H. (2010). Mouse ES cell culture system as a model of development. *Development, Growth & Differentiation*, *52*(3), 275–83. http://doi.org/10.1111/j.1440-169X.2009.01166.x

Noisa, P., Raivio, T., & Cui, W. (2015). Neural Progenitor Cells Derived from Human Embryonic Stem Cells as an Origin of Dopaminergic Neurons, *2015*. http://dx.doi.org/10.1155/2015/647437

Nolivos, S., Pages, C., Rousseau, P., Le Bourgeois, P., & Cornet, F. (2010). Are two better than one. Analysis of an FtsK/Xer recombination system that uses a single recombinase. *Nucleic Acids Research*, *38*(19), 6477–6489. http://doi.org/10.1093/nar/gkq507

Nordin, N., Li, M., & Mason, J. O. (2008). Expression profiles of Wnt genes during neural differentiation of mouse embryonic stem cells. *Cloning and Stem Cells*, *10*(1), 37–48. http://doi.org/10.1089/clo.2007.0060

Nusse, R., & Varmus, H. E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*, *31*(1), 99–109. http://doi.org/10.1016/0092-8674(82)90409-3

Nüsslein-Volhard, C., & Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. *Nature*, *287*(5785), 795–801. http://doi.org/10.1038/287795a0

Okada, Y., Matsumoto, A., Shimazaki, T., Enoki, R., Koizumi, A., Ishii, S., Okano, H. (2008). Spatiotemporal recapitulation of central nervous system development by murine embryonic stem cell-derived neural stem/progenitor cells. *Stem Cells*, *26*(12), 3086–3098. http://doi.org/10.1634/stemcells.2008-0293

Pankratz, M. T., Li, X,J., LaVaute, T. M., Lyons, E. A., Chen, X., & Zhang, S.C. (2007). Directed Neural Differentiation of Human Embryonic Stem Cells via an Obligated Primitive Anterior Stage. *Stem Cells*, *25*(6), 1511–1520. http://doi.org/10.1634/stemcells.2006-0707

Parish, C. L., Castelo-branco, G., Rawal, N., Tonnesen, J., Toft, A., Salto, C., Arenas, E. (2008). Wnt5a-treated midbrain neural stem cells improve dopamine cell replacement therapy in parkinsonian mice. *The Journal of Clinical Investigation, 118*(1). http://doi.org/10.1172/JCI32273.ES

Pauklin, S., Pedersen, R. A., & Vallier, L. (2010). ARTICLE SERIES : Stem Cells Pluripotency during early embryonic Mouse pluripotent stem cells at a glance. http://doi.org/10.1242/jcs.074120

Pevny, L. H., & Nicolis, S. K. (2010). Sox2 roles in neural stem cells. *International Journal of Biochemistry and Cell Biology*. http://doi.org/10.1016/j.biocel.2009.08.018

Pino, D., Choe, Y., & Pleasure, S. J. (2011). Wnt5a controls neurite development in olfactory bulb interneurons. *ASN Neuro*, *3*(3), e00059. http://doi.org/10.1042/AN20100038

Reinert, R. B., Kantz, J., Misfeldt, A. A., Poffenberger, G., Gannon, M., Brissova, M., & Powers, A. C. (2012). Tamoxifen-Induced Cre-loxP Recombination Is Prolonged in Pancreatic Islets of Adult Mice, *7*(3). http://doi.org/10.1371/journal.pone.0033529

Renström, J., Kröger, M., Peschel, C., & Oostendorp, R. A. J. (2010). How the niche regulates hematopoietic stem cells. *Chemico-Biological Interactions*. http://doi.org/10.1016/j.cbi.2009.11.012

Ribeiro, D., Laguna Goya, R., Ravindran, G., Vuono, R., Parish, C. L., Foldi, C., Arenas, E. (2013). Efficient expansion and dopaminergic differentiation of human fetal ventral midbrain neural stem cells by midbrain morphogens. *Neurobiology of Disease*, *49*(1), 118–127. http://doi.org/10.1016/j.nbd.2012.08.006

Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., & Nusse, R. (1987). The Drosophila homology of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell*, *50*(4), 649–657. http://doi.org/10.1016/0092-8674(87)90038-9

Rodda, D. J., Chew, J. L., Lim, L. H., Loh, Y. H., Wang, B., Ng, H. H., & Robson, P. (2005). Transcriptional regulation of Nanog by OCT4 and SOX2. *Journal of Biological Chemistry*, *280*(26), 24731–24737. http://doi.org/10.1074/jbc.M502573200

Rodda, S. J., Kavanagh, S. J., Rathjen, J., & Rathjen, P. D. (2002). Embryonic stem cell differentiation and the analysis of mammalian development. *The International Journal of Developmental Biology*, *46*(4), 449–58. http://www.ijdb.ehu.es/web/paper.php?doi=12141431

Ross, S. A, McCaffery, P. J., Drager, U. C., & De Luca, L. M. (2000). Retinoids in embryonal development. *Physiological Reviews*, *80*(3), 1021–1054. http:// doi:10.1152/physrev.2000.80.3.1021

Rosso, S. B., Sussman, D., Wynshaw-Boris, A., & Salinas, P. C. (2005). Wnt signaling through Dishevelled, Rac and JNK regulates dendritic development. *Nature Neuroscience*, *8*(1), 34–42. http://doi.org/10.1038/nn1374

Rowitch, D. H., & Kriegstein, A. R. (2010). Developmental genetics of vertebrate glial-cell specification. *Nature*, *468*(7321), 214–22. http://doi.org/10.1038/nature09611

Sadegh, C., & Macklis, J. D. (2014). Established monolayer differentiation of mouse embryonic stem cells generates heterogeneous neocortical-like neurons stalled at a stage equivalent to midcorticogenesis. *Journal of Comparative Neurology*, *522*(12), 2691–2706. http://doi.org/10.1002/cne.23576

Sanchez-Pernaute, R., Lee, H., Patterson, M., Reske-Nielsen, C., Yoshizaki, T., Sonntag, K. C., Isacson, O. (2008). Parthenogenetic dopamine neurons from primate embryonic stem cells restore function in experimental Parkinson's disease. *Brain : A Journal of Neurology*, *131*(Pt 8), 2127–39. http://doi.org/10.1093/brain/awn144

Sato, A., Yamamoto, H., Sakane, H., Koyama, H., & Kikuchi, A. (2009). Wnt5a regulates distinct signalling pathways by binding to Frizzled2. *The EMBO Journal*, *29*(1), 41–54. http://doi.org/10.1038/emboj.2009.322

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, *4*(1-2), 7–25.

Schulte, G., Bryja, V., Rawal, N., Castelo-Branco, G., Sousa, K. M., & Arenas, E. (2005). Purified Wnt-5a increases differentiation of midbrain dopaminergic cells and dishevelled phosphorylation. *Journal of Neurochemistry*, *92*(6), 1550–1553. http://doi.org/10.1111/j.1471-4159.2004.03022.x

Shafer, B., Onishi, K., Lo, C., Colakoglu, G., & Zou, Y. (2011). Vangl2 Promotes Wnt/Planar Cell Polarity-like Signaling by Antagonizing Dvl1-Mediated Feedback Inhibition in Growth Cone Guidance. *Developmental Cell*, *20*(2), 177–191. http://doi.org/10.1016/j.devcel.2011.01.002

Shen, M. M., & Leder, P. (1992). Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(17), 8240–4. http://doi.org/10.1073/pnas.89.17.8240

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: Biology and pathology. *Acta Neuropathologica,* 119(1), 7-35. http://doi.org/10.1007/s00401-009-0619-8

Soltani, M. H., Pichardo, R., Song, Z., Sangha, N., Camacho, F., Satyamoorthy, K., Setaluri, V. (2005). Microtubule-associated protein 2, a marker of neuronal differentiation, induces mitotic defects, inhibits growth of melanoma cells, and predicts metastatic potential of cutaneous melanoma. *The American Journal of Pathology*, *166*(6), 1841–50. http://doi.org/10.1016/S0002-9440(10)62493-5

Steinbeck, J. A., Koch, P., Derouiche, A., & Brüstle, O. (2012). Human embryonic stem cell-derived neurons establish region-specific, long-range projections in the adult brain. *Cellular and Molecular Life Sciences*, *69*(3), 461–470. http://doi.org/10.1007/s00018-011-0759-6

Ströbing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J., & Wobus, A. M. (1995). Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mechanisms of Development*, *53*(2), 275–287. http://doi.org/10.1016/0925-4773(95)00446-8.

Sun, X. Z., Takahashi, S., Cui, C., Zhang, R., Sakata-Haga, H., Sawada, K., & Fukui, Y. (2002). Normal and abnormal neuronal migration in the developing cerebral cortex. *J Med Invest*, *49*(3-4), 97–110.

Thomson, J. A., Itskovitz-eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic Stem Cell Lines Derived from

Human Blastocysts. *Science*, *282*(5391), 1145–1147.

Tripathy, D., Haobam, R., Nair, R., & Mohanakumar, K. P. (2013). Engraftment of Mouse Embryonic Stem Cells Differentiated by Default Leads to Neuroprotection , Behaviour Revival and Astrogliosis in Parkinsonian Rats. *Plos One, 8*(9), 1–13. http://doi.org/10.1371/journal.pone.0072501

Tropepe, V., Hitoshi, S., Sirard, C., Mak, T. W., Rossant, J., & Van Der Kooy, D. (2001). Direct neural fate specification from embryonic stem cells: A primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron*, *30*(1), 65–78. http://doi.org/10.1016/S0896-6273(01)00263-X

Tsuji, Y., Yoshimura, N., Aoki, H., Sharov, A. A., Ko, M. S., Motohashi, T., & Kunisada, T. (2008). Maintenance of undifferentiated mouse embryonic stem cells in suspension by the serum-and feeder-free defined culture condition. *Developmental Dynamics*, *237*(8), 2129-2138.. http://doi.org/10.1002/dvdy.21617.

Uccelli, A., Moretta, L., & Pistoia, V. (2008). Mesenchymal stem cells in health and disease. *Nature Reviews. Immunology*, *8*(9), 726–736. http://doi.org/10.1038/nri2395

Valamehr, B., Jonas, S. J., Polleux, J., Qiao, R., Guo, S., Gschweng, E. H., Wu, H. (2008). Hydrophobic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies Results. *Pnas*, *105*(38), 14459–14464. http://doi.org/10.1073/pnas.0807235105

Vallier, L., Alexander, M., & Pedersen, R. (2007a). Conditional gene expression in human embryonic stem cells. *Stem Cells (Dayton, Ohio)*, *25*(6), 1490–7. http://doi.org/10.1634/stemcells.2006-0825

Vallier, L., Alexander, M., & Pedersen, R. (2007b). Conditional gene expression in human embryonic stem cells. *Stem Cells*, *25*(6), 1490–1497. http://doi.org/10.1634/stemcells.2006-0825

Vallier, L., Mancip, J., Markossian, S., Lukaszewicz, A., Dehay, C., Metzger, D., Savatier, P. (2001). An efficient system for conditional gene expression in embryonic stem cells and in their in vitro and in vivo differentiated derivatives. *Proceedings of the National Academy of Sciences of the United States of America*, 98(5), 2467–2472. http://doi.org/10.1073/pnas.041617198

Van Amerongen, R., & Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. *Development (Cambridge, England)*, *136*(19), 3205–3214. http://doi.org/10.1242/dev.033910

Varela-Nallar, L., Alfaro, I. E., Serrano, F. G., Parodi, J., & Inestrosa, N. C. (2010). Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proceedings of the National Academy of*

Sciences, *107*(49), 21164–21169. http://doi.org/10.1073/pnas.1010011107/

Wan, W., Xia, S., Kalionis, B., Liu, L., & Li, Y. (2014). The role of Wnt signaling in the development of alzheimer's disease: A potential therapeutic target. *BioMed Research International*. http://doi.org/10.1155/2014/301575

Wang, Z., Oron, E., Nelson, B., Razis, S., & Ivanova, N. (2012). Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. *Cell Stem Cell*, *10*(4), 440–454. http://doi.org/10.1016/j.stem.2012.02.016

Weidinger, G., & Moon, R. T. (2003). When Wnts antagonize Wnts. *Journal of Cell Biology, 162*(5), 753-756. http://doi.org/10.1083/jcb.200307181

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*, *423*(6938), 448–452. http://doi.org/10.1038/nature01611\rnature01611

Wilson, L., Gale, E., & Maden, M. (2003). The role of retinoic acid in the morphogenesis of the neural tube. *Journal of Anatomy, 203*(4), 357-368.. http://doi.org/10.1046/j.1469-7580.2003.00230.x

Wobus, A. M., Kaomei, G., Shan, J., & Wellner, M. (1997). Retinoic Acid Accelerates Embryonic Stem Cell-Derived Cardiac Differentiation and Enhances Development of Ventricular Cardiomyocytes, *Journal of molecular and cellular cardiology*, *29*(6), 1525-1539 *1539*.

Yamaguchi, T. P. (2001). Heads or tails: Wnts and anterior-posterior patterning. *Current Biology*, *11*(17), R713-R724. http://doi.org/10.1016/S0960-9822(01)00417-1

Yamaguchi, T. P., Bradley, A., Mcmahon, A. P., & Jones, S. (1999). A Wnt5a pathway underlies outgrowth of multiple structures in the vertebrate embryo. *Development*, *126*(6), 1211-1223

Yamanaka, H., Moriguchi, T., Masuyama, N., Kusakabe, M., Hanafusa, H., Takada, R., Nishida, E. (2002). JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Reports*, *3*(1), 69–75. http://doi.org/10.1093/embo-reports/kvf008

Yan, Y., Shin, S., Jha, B. S., Liu, Q., Sheng, J., Li, F., Vemuri, M. C. (2013). Efficient and rapid derivation of primitive neural stem cells and generation of brain subtype neurons from human pluripotent stem cells. *Stem Cells Translational Medicine*, *2*(11), 862–70. http://doi.org/10.5966/sctm.2013-0080

Ying, Q.L., Stavridis, M., Griffiths, D., Li, M., & Smith, A. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nature Biotechnology*, *21*(2), 183–186. http://doi.org/10.1038/nbt780

Yu, J. M., Kim, J. H., Song, G. S., & Jung, J. S. (2006). Increase in proliferation and differentiation of neural progenitor cells isolated from postnatal and adult mice brain by Wnt-3a and Wnt-5a, 17–28. http://doi.org/10.1007/s11010-005-9113-3

Zaghetto, A. a, Paina, S., Mantero, S., Platonova, N., Peretto, P., Bovetti, S., Merlo, G. R. (2007). Activation of the Wnt-beta catenin pathway in a cell population on the surface of the forebrain is essential for the establishment of olfactory axon connections. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(36), 9757–9768. http://doi.org/10.1523/JNEUROSCI.0763-07.2007

Zhang, Y., & Xia, Y. (2012). Formation of embryoid bodies with controlled sizes and maintained pluripotency in three-dimensional inverse opal scaffolds. *Advanced Functional Materials*, *22*(1), 121–129. http://doi.org/10.1002/adfm.201101690

Zheng, B., Sage, M., Sheppeard, E. A., Jurecic, V., & Bradley, A. (2000). Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications. *Molecular and Cellular Biology*, *20*(2), 648–55. http://doi.org/10.1128/MCB.20.2.648-655.2000

Zhong, Z. A., Sun, W., Chen, H., Zhang, H., Lay, Y. A. E., Lane, N. E., & Yao, W. (2015). Optimizing tamoxifen-inducible Cre/loxp system to reduce tamoxifen effect on bone turnover in long bones of young mice. *Bone*, *81*, 614–619. http://doi.org/10.1016/j.bone.2015.07.034

Zhou, J., Xing, F., Shi, J., & Fang, Z. (2008). Quality of embryonic bodies and seeding density effects on neural differentiation of mouse embryonic stem cells, *32*, 1169–1175. http://doi.org/10.1016/j.cellbi.2008.04.025

Zhu, X., Ai, Z., Hu, X., Li, T., Liu, J. S., Hansen, D. V., Li, T. (2016). Efficient Generation of Corticofugal Projection Neurons from Human Embryonic Stem Cells. *Scientific Reports*, *6*(February), 28572. http://doi.org/10.1038/srep28572