LUDWIG INSTITUTE FOR CANCER RESEARCH and DEPARTMENT OF CELL & MOLECULAR BIOLOGY, Karolinska Institutet, Stockholm, Sweden

INTEGRATING EXTRINSIC AND INTRINSIC CUES TO GUIDE CELL FATE DECISIONS – RATIONAL APPROACHES IN STEM CELL ENGINEERING

Jamie Mong

Karolinska Institutet

Stockholm 2013

On cover: Mouse embryonic stem cell-derived noradrenergic neurons co-expressing the norepinephrine transporter (in red) and Phox2a (in green)

Published by Karolinska Institutet. Printed by Eprint AB 2013.

ISBN 978-91-7549-231-5

To my parents,

Mr and Mrs Mong Kum Jang

ABSTRACT

The long-term goal of stem cell engineering is to generate functional cells for cell replacement therapies, disease modelling and in drug development assays. It is evident that one of the major challenges in stem cell research is to develop reproducible methods to obtain well-defined and pure populations of clinically relevant cell types in a sustainable manner. Many studies have shown that appropriate signalling factors can specify desired cell types from stem cells, albeit in an inefficient manner. The heterogeneity seen in stem cell-derived cultures makes them unsafe and ineffective for use in the clinics and laboratories. In this thesis, we applied knowledge obtained from early developmental studies to develop rational approaches in stem cell engineering of a variety of clinically important cell types. In Paper I, we created mesendodermal progenitors by long-term activation of the Wnt pathway using a chemically defined inhibitor. These progenitors served as a renewable platform for more efficient stepwise derivation of cardiac, endothelial, osteogenic and chondrogenic cells. In Papers II and III, we integrated extrinsic and intrinsic cues by creating a permissive environment using appropriate growth factors, then forcing the expression of key transcription factors to achieve a highly efficient method to generate an array of neuronal cell types including dopamine, serotonin, motor and noradrenergic neurons. The purity of the cultures makes it possible to analyse subtype-specific genome-wide gene expression patterns and the discovery of novel markers provide insight into their transcriptional codes. We also showed, in a proof-of-concept experiment that the stem cell-derived neurons can be used in high throughput drug assays to analyse drug specificity.

LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

I. Manjiri Manohar Bakre, Aina Hoi, **JAMIE CHEN YEE MONG**, Yvonne Yiling Koh, Kee Yew Wong, Lawrence W Stanton. (2007). Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. The Journal of Biological Chemistry 282(43):31703-12

II. Lia Panman*, Elisabet Andersson*, Zhanna Alekseenko#, Eva Hedlund#, Nigel Kee#, **JAMIE MONG**#, Christopher W Uhde#, Qiaolin Deng#, Rickard Sandberg, Lawrence W Stanton, Johan Ericson, Thomas Perlmann. (2011). Cell Stem Cell 8(6):663-75

*These authors contributed equally to this work
#These authors contributed equally to this work (by alphabetical order)

III. **JAMIE MONG**, Lia Panman, Zhanna Alekseenko, Nigel Kee, Lawrence W. Stanton, Johan Ericson and Thomas Perlmann. (2013). Manuscript in revision.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AADC	Aromatic L-amino acid decarboxylase
AHD2	Aldehyde dehydrogenase 2
Aldh	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
BIO	6-bromoindirubin-3'-oxime
BMPs	Bone morphogenetic factors
CNS	Central nervous system
DAN	Dopamine neurons
DAT	Dopamine transporter
Dbh	Dopamine beta-hydroxylase
Dkk1	Dickkopf1
ESCs	Embryonic stem cells
FGF8	Fibroblast growth factor 8
GSK-3	Glycogen synthase kinase 3
i-GSK3β	Inhibitor of glycogen synthase kinase 3β
iPSCs	Induced pluripotent stem cells
LC	Locus coeruleus
LIF	Leukaemia inhibitory factor
MN	Motor neurons
MPC	Mesendodermal progenitor clones
NAN	Noradrenergic neurons
PD	Parkinson’s disease
PSA-NCAM	Polysialylated-neural cell adhesion molecule
RA	Retinoic acid
SN	Serotonergic neurons
Shh	Sonic hedgehog
TH	Tyrosine kinase
VE	Visceral endoderm
VMAT	Vesicular monoamine transporter
vMN	Visceral motor neurons

1 INTRODUCTION

1.1 What is stem cell engineering? Why stem cell research?

In my own words, stem cell engineering is the application of *in-vivo* knowledge to derive clinically-relevant cell types from "stem" cells, in an efficient and sustainable manner (inspired by the definition of "engineering" by the American Engineer's Council). Stem-ness is an ability associated with the cell's multipotency (the potential to give rise to its descendents) and its ability to self-renew. It is easy to see why stem cell engineering has important applications in therapy – the most straightforward of all in regenerative medicine.

1.1.1 Stem cells in regenerative medicine

In regenerative medicine, we restore normal function in a disorder by replacing or regenerating diseased or lost cells. Unlike the salamander that possesses the natural ability to regenerate complex structures such as their limbs, tail, neurons, etc, humans have a limited capacity for regeneration (Morrison et al., 2006; Parish et al., 2007).

In disorders whereby cells are diseased or lost for yet unknown/unstoppable reasons (e.g. Parkinson's disease (PD), spinal cord injury, type I diabetes, heart disease, etc), cell replacement therapy provides a superior alternative to pharmacological agents for symptomatic relief. For instance, drugs such as L-DOPA in PD, or insulin in diabetes are not delivered directly to the sites of action and their doses are not titrated according to the specific needs of the body; Implanted neurons or pancreatic beta cells integrate inputs from their surroundings and deliver appropriate levels outputs to specific targets at the appropriate times, minimising side effects seen in therapy by pharmacological agents.

In the clinics today, we already see bone marrow stem cell transplants in patients with leukaemia as a life-saving procedure. Neurodegenerative diseases represent a plethora of disorders in which therapies are only available for symptom relief, and the progressive nature of the disease eventually renders most therapies ineffective. At the onset of PD, for example, more than 80% of the dopamine neurons would have been lost. In 1986, scientists in Sweden began small clinical trials to transplant ventral mesencephalic tissue from aborted foetuses into PD patients (Lindvall et al., 1990). The grafted tissue survived and showed signs of innervations, leading to promising improvements in some of the patients (Lindvall et al., 1989; Lindvall et al., 1992). The results from subsequent larger clinical trials using foetal

material ranged from promising to being clinically insignificant and were not convincing enough to bring cell replacement therapy to the clinics (Freed et al., 1992; Freed et al., 2001; Hagell et al., 1999; Olanow et al., 2003; Spencer et al., 1992; Widner et al., 1992). In retrospect, this was not surprising because there were, however, glaring obstacles in such procedures, the most frustrating of all is contributed by variability in preparations of donor tissues used which is already limited in availability (Barker et al., 2013; Bjorklund and Kordower, 2013; Politis et al., 2011).

Unlike foetal tissue, which are subjected to ethical issues and are limited in availability, ESC-derived neurons represent a potentially unlimited source of neurons that can not only be used in transplants, but also as cellular models to understanding disease mechanisms or in drug assays. Cells can be generated in a dish under good manufacturing practice conditions so that they are safe for human use. The potential for stem cell as replacement therapy also applies for other diseases with a degenerative nature such as in diabetes, or in organ failures such as heart failures, etc.

With advances in surgical techniques, imaging and infection control, the rate-limiting step now lies in finding methods to derive an efficient and reliable source of well-characterised and functional cells for replacement therapy.

1.1.2 Stem cells in disease modelling

It has become increasingly apparent that cell lines traditionally used to understand physiological processes or for toxicity/drug screening are limited in their capacity to mimic the *in-vivo* situation. Commonly used ovarian cancer cell lines, for instance, have significantly different mutation and gene expression profiles from what is observed in ovarian tumours (Domcke et al., 2013). Cell lines of neuronal origin such as PC12 express neural receptors, react to nerve growth factors and even produce neurotransmitters such as the catecholamine. Although easy to use, these tumour-derived immortalized cell lines (PC12 from the rat pheochromocytoma) have abnormal genotypes and their genomic instability makes them vulnerable to selection pressures. It is possible to carefully characterise cell lines before use but there is still a lack of cell lines that represent the myriad of neuronal subtypes present in the nervous system. Immortalised hepatocyte cell lines are a valuable resource for studying drug-induced hepatotoxicity but they are plagued with problems of genetic instability and poor resemblance to *in-vivo* function (Dalgetty et al., 2009; Delgado et al., 2005; Tachibana et al., 2011).

ESC-derived cells represent a biologically relevant representation of their in-vivo counterparts. ESCs possess the potential to give rise to all cell types of the adult human body and hence is a source of any cell types needed for study. With the advent of the induced pluripotent stem cells (iPSCs), which I will elaborate in a later section, stem cell engineering has gained an additional niche in disease modelling of developmental disorders with a genetic basis. iPSCs may be the answer to ethical and incompatibility issues in heterologous transplants, although it may not be the wisest to use material from the same patients who carry the disease-causing genetic defect. Such material, however, has unprecedented potential in disease modelling. iPSCs have the ability to recapitulate the original genetic defects seen in such patients, providing means to analyse, *in-vitro,* disease mechanisms or to act as tools for drug screening. By coaxing iPSCs from patients to develop into the clinically relevant cell types (patient-specific iPSCs), we can observe, in a dish, processes that go wrong during their early development and possibly find ways to correct them. iPSCs derived from Rett syndrome patients, for example, can be differentiated into neurons and these neurons not only behaved differently, but were also smaller in size (Kim et al., 2011b; Marchetto et al., 2010). In neurodegenerative diseases where the underlying cause is not as well understood, such as idiopathic Parkinson's disease, neurons derived from patient-specific iPSCs allow us to study cell types that are otherwise lost in the disease (Sanchez-Danes et al., 2012b). iPSCs derived from patients with familial or sporadic Alzheimer's disease gave rise to neurons and astrocytes that accumulated amyloid peptides, leading to increased oxidative stress (Kondo et al., 2013). Such *in-vitro* systems are particularly important for diseases which lack good animal models to study disease mechanisms.

Taking a step further than merely disease modelling, several groups have explored the use of iPSCs in correcting the underlying pathological processes. As a proof-of-principle for the potential to cure diseases with the aid of iPSCs and stem cell engineering, a group made iPSCs from mice models of sickle cell anaemia, corrected the mutation in the haemoglobin gene *in-vitro*, then differentiated the iPSCs into blood forming progenitors that goes on to repopulate the blood with normal red blood cells, hence curing the mouse (Hanna et al., 2007). This has very quickly caught on in similar experiments in patient-specific human iPSCs with novel strategies to correct the site-specific mutation (Sebastiano et al., 2011; Zou et al., 2011).

1.1.3 Stem cells in toxicity/drug screening

Besides using these *in-vitro* engineered cells for transplants, stem cell-derived specialised cells can be used in high-throughput drug screening platforms to complement complicated and expensive animal testing. Cardiac toxicity, in particularly drug-induced arrhythmia, is one of the key concerns in assessing the safety of a new drug compound (Kraushaar et al., 2012). There are now extensive studies to assess the suitability of stem cell-derived cardiomyocytes to replace primary cultures or non-cardiogenic cell lines in large-scale *in-vitro* assays for cardiotoxicity screens (Abassi et al., 2012; Kettenhofen and Bohlen, 2008; Ma et al., 2011). Recently, a group has created a library of patient-specific iPSC from patients with pre-existing heart disease and showed that such iPSC-derived cardiomyocytes recapitulates the drug-induced cardiotoxicity profiles seen in the different groups of patients (Liang et al., 2013).

Other than toxicity screens, stem cells provide an avenue for us to derive in a controlled and high-throughput manner, disease- or patient-specific cells to screen for drug candidates. Amyotrophic lateral sclerosis patients suffer from degenerating motor neurons that is eventually fatal. Motor neurons engineered from patient-specific iPSC can be used to screen for chemical compounds that rescue the phenotype, and this was shown to be fruitful in the discovery of anacardic acid as a candidate drug (Egawa et al., 2012).

The key to success in all the endeavours described above lies in efficient, reproducible methods to drive the differentiation of pluripotent stem cells into clinically relevant cell types that are homogenous and functional.

1.2 Introducing the embryonic stem cell (ESC) and induced pluripotent stem cells (iPSCs)

1.2.1 What is the embryonic stem cell (ESC)?

In 1981, Evans and Kaufman (Evans and Kaufman, 1981) and Martin (Martin, 1981) described the isolation of the inner cell mass from mouse blastocysts and established in culture under specific conditions, embryonic stem cells (ESC) that are self-renewing and retains the potential to give rise to cells of the three germ layers, either as teratocarcinomas when transplanted in a mouse, or as outgrowths from rounded aggregates of cells (embryoid bodies) when plated on a dish. Such outgrowths

were comprised of a random mixture of cell types from the three germ layers. When ESCs were derived from human blastocysts in 1998 (Thomson et al., 1998), it became even more apparent that ESC research is clinically relevant in terms of regenerative medicine and drug screening and opened the floodgates to ESC research.

The ground state of pluripotency of an embryonic stem cell is often defined experimentally by (but not limited to)

- Gene expression of *Oct4, Sox2, Nanog*, amidst an array of other markers such as *Klf4* and *Rex1*. Conversely, lack of expression of markers of the three germ layers such as *Foxa2, Sox17, Gata4* (endoderm); *T, Hand1* (mesoderm); *Sox1*, *Sox3, Pax6*(ectoderm).
- Appearance of cells with a large nuclear-to-cytoplasmic ratio in compact, rounded colonies.
- Positive staining for alkaline phosphatase.
- Long-term self-renewal *in-vitro*.
- Differentiates *in-vitro* (forms embryoid bodies) and *in-vivo* (forms teratomas) to give rise to cells representative of the three germ layers.
- Ability to significantly contribute to chimeras.

Despite the common theme revolving around *Oct4, Sox2* and *Nanog* in governing the pluripotency network of mouse and human ESCs, there are significant differences in the signalling pathways involved. Pluripotency of mouse ESCs depends on leukaemia inhibitory factor (LIF) and bone morphogenetic factors (BMPs) (Ying et al., 2003), whereas human ESCs require activin and fibroblast growth factor (FGF). Mouse ESCs express the surface marker SSEA-1 while human ESCs express SSEA-4.

Such differences motivated researchers to look for an ESC model that is closer to their human counterpart and this has led to the discovery of epiblast stem cells (EpiSCs) from the mouse in 2007 (Brons et al., 2007). Unlike ESCs, which are obtained from the ICM at earlier pre-implantation stages, EpiSCs are obtained from post-implantation epiblast cells just before gastrulation (E5.5-7.5). Like ESCs, they express Oct4, Sox2 and Nanog and have the ability to give rise to cells of the three germ layers both *in-vitro* and *in-vivo*. Unlike mouse ESCs, mouse EpiSCs more closely resemble human ESC in terms of their appearance (larger colonies in a monolayer), response to activin and FGFs in culture to maintain pluripotency (and the lack of response to LIF) and the pluripotency transcriptional network (Tesar et al., 2007). Due to their similarities to the human ESC in ways unmatched by the mouse ESCs, they are

suggested to be a more clinically relevant model to study about stem cell pluripotency in the mice. It is hence important for the stem cell biologist to keep in mind inherent differences between ESCs of different species (on top of differences between various cell lines), and to reconfirm studies seen in mouse ESCs in their human counterparts.

Today, however, probably due to the extensive resources already invested in ESCs in stem cell research and their greater potential associated with their primitive state (as compared to EpiSCs), the mouse ESC still remains the more popular cell line in stem cell differentiation studies over mouse EpiSCs. Despite the lack of clinical relevancy, mouse ESCs still present with advantages over human ESCs in certain aspects of research. For example, in developmental studies, it is easy to determine the biological relevance of mouse ESC-derived neurons in the mouse embryonic brain by large-scale in-situ hybridisation studies, or even in transplantation studies (Paper II and III) (Friling et al., 2009a; Panman et al., 2011). The work in this thesis is largely based on the mouse ESC line E14.

1.2.2 Induced pluripotent stem cells (iPSCs)

iPSCs at the very beginning refers to fibroblast cells that were "induced" to become pluripotent stem cell-like by the lentiviral overexpression of three transcription factors that form the core of pluripotency – *Oct4, Sox2 and Klf4*, as well as *c-Myc*, first in mice (Takahashi and Yamanaka, 2006), then in the humans (Takahashi et al., 2007; Yu et al., 2007). The field has very quickly expanded to include an array of somatic cells – including adult neural stem cells, cardiomyocytes, blood cells, etc that could be similarly induced to display properties of ESCs (Aoi et al., 2008; Kim et al., 2008; Staerk et al., 2010). Other combinations of factors have been reported to similarly induce pluripotency including *Oct4/Sox2/Nanog/Lin28* (Yu et al., 2007), *Nr5a2/Sox2/Klf4* (Heng et al., 2010), *Oct4/Sox2/Esrrb* (Feng et al., 2009), etc. The original set of four factors has also been reduced to three (Nakagawa et al., 2008) (since the oncogenic *c-Myc* is non-compatible for clinical uses) or even to just one (Kim et al., 2009a), depending on the cell context. iPSCs can be differentiated to a variety of clinically relevant cell types including cardiomyocytes, neurons, blood cells, hepatocytes, etc using the same protocols that were first discovered using ESCs (Kondo et al., 2013; Lan et al., 2013; Si-Tayeb et al., 2010; Takebe et al., 2013).

However, not surprisingly, there are increasing evidences that different iPSC lines are not identical and that they may not be exactly the same as human ESCs in terms of methylation status, genome-wide gene expression and propensity to

differentiation (Chin et al., 2009; Kim et al., 2011a; Lowry, 2012; Ohi et al., 2011; Sandoe and Eggan, 2013). Whether or not such molecular differences will result in functional distinctions will require further investigations. What is relevant to the stem cell engineer is to keep such differences in mind in planning experiments and to use them to our advantage. For instance, residual epigenetic memory from their former self may aid differentiation to the same lineage (Bar-Nur et al., 2011; Kim et al., 2011a). This means that in theory, we can derive neurons more efficiently from iPSCs made from neural tissue. We should also include multiple control lines in differentiation studies to make solid conclusions (Sandoe and Eggan, 2013).

1.3 Lessons from developmental biology in cell fate decisions- What are "Extrinsic" and "Intrinsic" cues?

Since ESCs are derived from the inner cell mass, which is then primed to differentiate and to give rise to the three germ layers, it is important to understand the early developmental processes that guide cell fate decisions *in-vivo* so that we can recapitulate the process *in-vitro*. We can see from developmental studies the recurring theme of extrinsic cues in the form of growth factors to induce intrinsic cues in the form of transcription factors, which, in turn, leads to and stabilises the phenotype together with extrinsic survival cues.

1.3.1 Early development of the vertebrate embryo

When an oocyte is fertilized by a sperm, it kickstarts a series of mitotic divisions to give rise to a ball of cells which enlarges as it moves towards the uterus. The first differentiation event occurs by the 64-cell stage, whereby the inner cell mass and the outer trophoblast cells become separate cell layers. The inner cell mass gives rise to the embryo and its associated yolk sac, allantois and amnion while the trophoblast cells give rise to the placenta and produces enzymes and cell adhesion molecules critical for the implantation of the embryo into the uterine wall (Gilbert et al., 2000).

After implantation, the inner cell mass segregates to become the visceral endoderm (hypoblast) and the epiblast. In a process termed gastrulation, the embryonic epiblast cells forms the three germ layers that makes up the body of the adult animal – the ectoderm, mesoderm and endoderm. Just before gastrulation, the stage is set with

the formation of the extraembryonic anterior visceral endoderm, which secretes Wnt and Nodal antagonists (Dkk1 and Lefty1) to mark the anterior pole of the embryo. During gastrulation, Wnt and Nodal signals at the posterior end induces the formation of the primitive streak along the midline, extending anteriorly to the node. The epiblast layer (what is now known as the ectodermal layer) anterior to the primitive streak thickens and folds to form the neural tube in response to signals from the underlying notochord and the anterior visceral endoderm (Gilbert et al., 2000). This process is known as neurulation, which will be elaborated in a later section. At the same time, cells divide and migrate through the primitive streak first downwards, then laterally and anteriorly, to give rise to two new layers below - the definitive endoderm and the mesoderm. In the xenopus and zebrafish embryos (Rodaway and Patient, 2001), there is a bipotent layer of cells that gives rise to both the mesoderm and the endoderm, hence referred to as the mesendoderm. In mouse embryos, fate mapping (Kinder et al., 2001; Lawson et al., 1991) points to cells present in the organizers (from the primitive streak) that give rise to the anterior definitive endoderm and axial mesoderm. Studies using embryonic stem cells (Kubo et al., 2004; Tada et al., 2005) have also shown that in differentiation cultures, brachyury or goosecoid-expressing cells have the potential to give rise to both the endoderm and the mesoderm.

In the adult animal, the ectoderm contributes to the epidermis, teeth, the nervous system, etc; The mesoderm develops into muscles, connective tissues, blood, bone, heart and gonads; The definitive endoderm forms the liver, pancreas, bladder and the lining to lungs and the digestive gut. As such, the inner cell mass is described to be "pluripotent" – to have the potential to give rise to all cells of the adult body.

Paper I describes the application of extrinsic cues through the canonical Wnt pathway in selecting for mesendodermal cell fates from ESCs. Such mesendodermal progenitor clones are not only self-renewable and reversible, but also show a propensity to differentiate to the endothelial, cardiac, osteogenic, and chondrogenic lineages. Paper II and III explores biologically-relevant external signals that provides a permissive environment for clinically important neuronal subtypes to arise, then integrates extrinsic signals with intrinsic signals in the form of transcription factors to improve differentiation efficiencies of ESCs.

1.3.2 A simplified view on neurulation and neuronal specification

At its very beginnings, the nervous system starts out as a tubular structure known as the neural tube. Following gastrulation, neurulation occurs in which presumptive epidermal cells at the two edges of the neural plate move towards each other, folding the neural plate and pushing the edges of the neural plate together so that they close up to form the neural tube. Cells of the neural tube eventually give rise to the neurons, astrocytes and oligodendrocytes that make up the nervous system (Gilbert et al., 2000).

I will focus on the description of the anterior parts of the neural tube that eventually make up the brain. After the neural plate joins at its anterior ends, the tube swells to give rise to three primary brain vesicles – the forebrain, midbrain and hindbrain, which goes on to become more finely divided into five secondary vesicles – Telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. Specification of these vesicles, as well as the Nestin-positive neuronal progenitor cells that line the vesicles are guided by extrinsic signals from the underlying mesoderm (retinoic acid), notochord (sonic hedgehog) and epidermis (bone morphogenetic factors). Once the vesicles are properly setup, structures such as the isthmus at the mid-hindbrain boundary also acts to propagate the positional signal (fibroblast growth factor 8) for appropriate neuronal subtypes to be born (Gilbert et al., 2000).

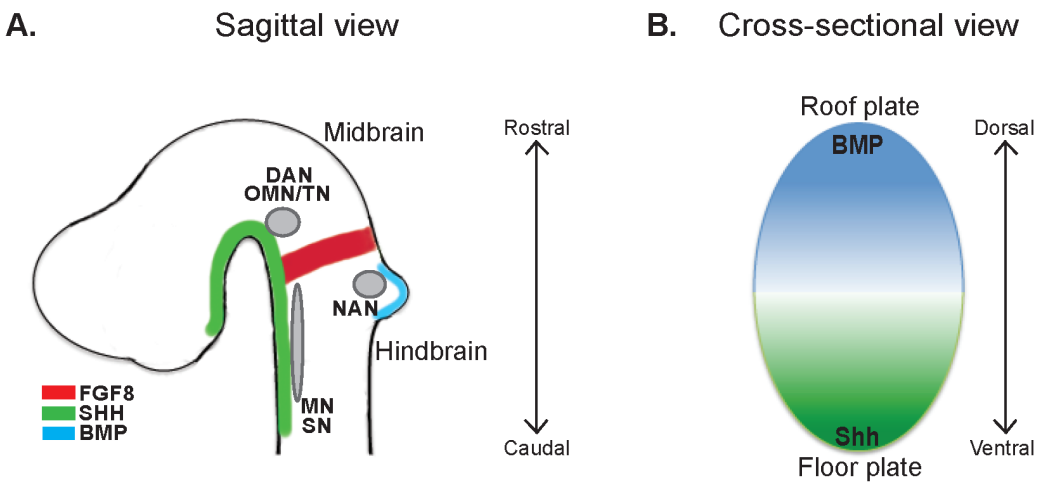

Figure 1: Schematic of the mouse embryonic brain. (A) Sagittal view of positions of neuronal subtypes described in Papers II and III. Mesencephalic dopamine neurons (DAN) and oculomotor/trochlear neurons (OMN/TN) are born in the midbrain while noradrenergic neurons (NAN) and serotonergic neurons (SN) are born in the hindbrain. Some visceral motor neurons (vMN) are also born in the hindbrain. Neuronal subtype specification is influenced by growth factors in the vicinity of developing neurons such as FGF8 (red) in the isthmus, BMPs (blue) in the roof plate and sonic hedgehog, Shh (green) from the notochord. Note that only the mid- and hind-brain regions are considered in this schematic. (B) Cross-sectional view of the neural tube illustrates the gradient of Shh from the ventral floor plate and BMPs from the dorsal roof plate.

A number of clinically-relevant neural subtypes are born in the midbrain and hindbrain regions - dopamine neurons (DANs), motor neurons (MNs), serotonergic neurons (SNs), noradrenergic neurons (NANs). (Refer to Figure 1 for details). Other than NAN, which are born in dorsal rhombomere 1, the rest of them are found in the ventral half of the neural tube. From developmental studies in chick and mouse, we observe two factors key to their specification – The exposure to positionally appropriate environmental cues at a timely fashion (spatiotemporal), both of which can be manipulated *in-vitro* to drive cell fate specification from ESCs.

1.3.3 The roles of signalling molecules in specification

Signalling molecules in dorsal-ventral patterning of the neural tube

The graded response to morphogens and its interpretation into cell fate decisions is perhaps best illustrated by studies on the action of sonic hedgehog (Shh) in the specification of ventral neurons in the chick spinal cord. *In-vivo*, Shh from the notochord induces the formation of the floor plate, which in turn takes over the secretion of Shh. Shh diffuses across the neural tube, setting up a gradient of Shh exposure that induces the differentiation of ventral neuronal subtypes (Figure 1B) (Chiang et al., 1996; Yamada et al., 1991). As such, the more ventral is the neural progenitor, the greater is the exposure to Shh. In fact, varying Shh concentrations on *ex-vivo* cultures produces the five classes of ventral neurons in the spinal cord in a predictable fashion, based on their dorso-ventral positions in the neural tube (Chiang et al., 1996; Ericson et al., 1996; Marti et al., 1995; Placzek et al., 1991). Shh signal then induces the expression of relevant transcription factors, which goes on to stabilise the phenotype by cross-repressive interactions and feed-forward amplification or repression of the external signal. Such integration of extrinsic and intrinsic cues establishes the sharp borders between different classes of neurons (Briscoe and Ericson, 2001; Briscoe et al., 2000; Ericson et al., 1997; Lek et al., 2010; Oosterveen et al., 2012). Molecularly, Shh action is mediated through the Gli family of transcription factors (Ding et al., 1998).

In the dorsal third of the neural tube, the roof plate acts as another important organizer that secretes bone morphogenetic factors (BMPs) to induce the specification of dorsal neurons (Lee et al., 1998; Liem et al., 1997; Panchision et al., 2001). There are a few members in the family of BMPs (for instance, BMP6, 7 and Gdf7) secreted by the roof plate depending on the species (chick or mouse), the region (hindbrain

versus spinal cord) as well as on the timing. In a similar fashion as Shh, BMPs also sets up a transcriptional code in dorsal neurons (Timmer et al., 2002). In the specification of locus coeruleus (LC) NANs in the mouse hindbrain, BMP 5, 6 and 7 are expressed in positions close to where the Phox2a-positive noradrenergic precursor cells are born (Tilleman et al., 2010). However, these NANs are lost only when both BMP5 and BMP7 are deleted in the mouse, suggesting functional redundancy between BMP5 and BMP7 in noradrenergic development (Solloway and Robertson, 1999; Tilleman et al., 2010).

Signalling molecules in anterior-posterior patterning of the neural tube

Studies in the chick embryos reveal that the initial default identity of cells in the neural tube belongs to the rostral character and that caudal cells are induced by signalling molecules from a few key organizers (Muhr et al., 1999). The same phenomenon is observed in ESC neural differentiation in that ESCs cultured simply in neural differentiation media (N2/B27 media) expressed forebrain markers (Gaspard et al., 2009).

The isthmus (also referred as the mid-hindbrain boundary) provides important positional information along the rostral-caudal axis for the specification of neurons in the midbrain and rostral hindbrain. The actions of the isthmus is mediated largely by fibroblast growth factor 8 (FGF8) (Gilbert et al., 2000). FGF8 (Figure 1; FGF8 in red) has been shown to be key in the specification of a number of neural subtypes in the vicinity, such as DANs, MNs, rostral SN as well as NANs (Hidalgo-Sanchez et al., 1999; Liu et al., 1999).

Posteriorly, retinoic acid (RA) from the paraxial mesoderm has profound effects on the development of the caudal hindbrain. Quail hens put under a complete vitamin A-deficient diet and developing chick embryos with inhibited RA receptors present with misspecification of the posterior hindbrain regions such that they adopt a more anterior identity (Dupe and Lumsden, 2001; Maden et al., 1996; van der Wees et al., 1998). In neural tube specification, the more posterior the region, the greater is the requirement for RA (Dupe and Lumsden, 2001; Glover et al., 2006). RA is necessary for the specification of caudal hindbrain and spinal cord and inhibits midbrain and rostral hindbrain formation (Muhr et al., 1999; Niederreither et al., 2000). In ESC differentiation protocols, RA is a strong inducer for the neural lineage. We have observed, together with many others, that adding RA to ESCs accelerates cell cycle exit and the acquisition of neuronal identity (Kim et al., 2009b). In driving neural subtype

identity, exposure to higher RA concentrations means that the neuronal cells derived will express markers of the posterior hindbrain and spinal cord, such as the somatic motor neurons (Li et al., 2005; Panman et al., 2011).

For the forebrain to rostral hindbrain regions, Wnts from the paraxial mesoderm (together with FGF8) seem to be providing information in a dose-dependent manner in rostrocaudal axis specification (Muhr et al., 1999; Nordstrom et al., 2002). The Wnt signalling pathway is also key to the dorsal-ventral patterning of the neural tube. An overview to the Wnt signalling pathway will be provided in a later section and I will discuss further the effects of Wnt signalling on neural specification.

1.3.4 The canonical Wnt pathway in brief

The highly conserved Wnt family of secreted proteins has roles in germ layer and axis specification in early vertebrate development. Central to the action of the canonical Wnt signalling pathway is beta-catenin (encoded by the *Ctnb1* gene) and the TCF transcription factors(Alberts, 2008; Gilbert et al., 2000). Wnt proteins described in this thesis that signal through the canonical Wnt pathway include Wnt1 and Wnt3a.

Beta-catenin binds to the transmembrane cadherins at adherens junctions to regulate cell adhesion, and interacts intracellularly with alpha-catenin to regulate cell structure. Excess beta-catenin in the cytoplasm is phosphorylated and trapped in a destruction complex made up of glycogen synthase kinase (GSK-3), Axin, adenomatous polyposis coli (APC), and several other players. The canonical Wnt pathway is activated when Wnt proteins bind to its receptor Frizzled and co-receptors Lrp5/6. This in turn recruits Axin and activates Dishevelled (which inhibits GSK-3), destabilising the destruction complex and leaving beta-catenin dephosphorylated and intact. Beta-catenin is then free to translocate into the nucleus to cooperate with TCF/LEF transcription factors and activate transcription of Wnt target genes (Alberts, 2008; Gilbert et al., 2000). Other than Wnt proteins, chemical inhibitors of GSK-3 are commonly used in experiments to activate the Wnt pathway. GSK-3 inhibition, however, has other less well-known effects including regulating hedgehog signalling, transcription, microtubule synthesis and apoptosis. Different GSK-3 inhibitors also target different regions for inhibition. (Doble and Woodgett, 2003; Forde and Dale, 2007)

In vertebrates, there are four members in the TCF protein family including TCF1, LEF1, TCF3 and TCF4. Different TCFs are found in different embryonic tissues while all of the TCF family members are present in mouse embryonic stem

cells. TCF3 predominently represses transcription while LEF1, TCF3 and TCF4 reportedly activate transcription (Cadigan and Waterman, 2012).

The Wnt pathway in early embryonic development

During gastrulation, Wnt signalling is active in the visceral endoderm (VE). The anterior end of the VE secretes Dkk1 and Lefty1 to inhibit Wnt and Nodal signalling respectively. At the posterior end, Wnt and Nodal signalling remains active for the primitive streak to form. This event underlies the basis of anterior-posterior axis patterning in the developing embryo. In embryos lacking Wnt3 or beta-catenin, the primitive streak and the mesoderm do not form. Antagonising Wnt signalling is hence critical at the anterior end of the embryo for the development of the heart and the forebrain.

The Wnt pathway in embryonic stem cell pluripotency/differentiation

Activation of the Wnt pathway is reported to maintain pluripotency of mouse and human ESCs (Sato et al., 2004; ten Berge et al., 2011; Wray et al., 2011; Ying et al., 2008) and at the same time, to initiate mesendodermal differentiation (Bakre et al., 2007; Davidson et al., 2012; Sumi et al., 2008) and repress neuroectodermal differentiation (Aubert et al., 2002; Cajánek et al., 2009; Slawny and O'shea, 2011). Both ESCs and mouse embryonic fibroblasts commonly used in co-culture express Wnts (Sato et al., 2004; ten Berge et al., 2011). Wnt signalling was also described to be downregulated upon differentiation (ten Berge et al., 2011).

Beta-catenin's critical role in cell adhesion also adds to the complexity of the action of Wnt pathway in stem cell pluripotency. The appearance of compact, rounded colonies is one of the hallmarks of pluripotency in ESC, and this is maintained by interactions between beta-catenin, alpha-catenin and E-cadherins to result in tight cell adhesion. It has been reported that Ctnnb-deficient mouse ESCs maintain self-renewal properties but fail to give rise to the three germ layers properly (Lyashenko et al., 2011). However, another study observed a downregulation of pluripotency markers in the same Ctnnb(-/-) mouse ESCs, which can be rescued by preserving the cell adhesive function of beta-catenin (Del Valle et al., 2013).

The effects of Wnt on embryonic stem cells appears conflicting at a glance but they are not mutually exclusive. Due to Wnt's involvement in a multitude of early embryonic lineage decisions as discussed above, the effects seen *in-vitro* is dependent on the cell context, length of treatment and molecules used to activate the Wnt pathway

(Wnt3a protein/Chemical inhibitors). At the molecular level, such variability translates into numerous possibilities in the interplay of TCFs involved. Adding to the complexity is the multitude of players involved in pluripotency/differentiation decisions including BMPs, LIF/Stat3 and FGF/ERK (Ying et al., 2008). In our study, we observed that long-term activation of the Wnt pathway in mouse ESCs increased their propensity to differentiate to mesendodermal progenitors. I will discuss these seemingly contradictory effects of Wnt in greater details in the results section for Paper I.

The Wnt pathway in neural specification

The inhibitory effects of Wnt on neural induction in ESCs does not preclude its use in neural differentiation of ESCs. As memtioned in an earlier section, the Wnt signalling pathway has roles in both the rostrocaudal and dorsoventral patterning of the neural tube.

Wnt1 and Wnt3a proteins are expressed in the developing mouse roof plate (Summerhurst et al., 2008). The combined loss of Wnt1 and Wnt3a leads to a reduction in the number of dorsal interneurons in the developing chick spinal cord (Muroyama et al., 2002). Dickkopf1 (Dkk1) acts as a Wnt antagonist and Dkk1 null mouse mutants do not develop structures rostral to the midbrain (Mukhopadhyay et al., 2001).

In a different cellular context in the mouse ventral midbrain, Wnt1 and Wnt5a are reported to be required for the proliferation and differentiation of DANs, while Wnt3a enhances proliferation of dopamine neuronal progenitors (Andersson et al., 2008; Andersson et al., 2013; Castelo-Branco et al., 2003; Prakash et al., 2006). This is partly due to Wnts' requirement in the midbrain to inhibit Shh in the floor plate to allow DAN neurogenesis to occur (Joksimovic et al., 2009). In ESCs neural differentiation protocols, Wnt proteins (Wnt1, Wnt5a) are added together or after neural induction by other molecules (e.g. Noggin), to enhance the derivation of DANs (Andersson et al., 2013; Kirkeby et al., 2012; Ribeiro et al., 2012). Kirkeby and colleagues activated the Wnt pathway in human ESC-derived neural progenitors using a GSK-3 inhibitor and showed that by varying its dosage, it is possible to control the positional (rostral-to-caudal) identity of the neurons generated (Kirkeby et al., 2012).

Extrinsic signals in the developing embryo are managed tightly by constraints of timing, space, concentrations and interactions with the environment such that signals that induce a multitude of different neural subtypes (e.g. Wnts) at different positions can specifically instruct the birth of a specific neuron at a specific place. *In-vitro*

protocols that mimic *in-vivo* conditions often lack the full set of instructions required, hence leading to non-homogenous cultures in ESC differentiation. The context-dependent and ubiquitous nature of the actions of the Wnt pathway also highlights the limitations in directing stem cell differentiation merely by manipulating signalling molecules. In the next section, I will discuss the strategy we used to overcome such limitations in neuronal cell fate specification using transcription factors.

1.3.5 Transcription factors in neuronal identity specification

In this section I will give a brief overview on the developmental requirements of two neuronal subtypes described in this thesis (midbrain DAN and hindbrain NAN), focusing on the transcription factors that are key to their specification. Transcription factors belong to a group of proteins that bind to specific DNA sequences to transmit information from DNA to mRNA, which is subsequently translated into proteins (the final effector of cell processes). Transcription factors can work singly, or in combination with other transcription factors or cofactors, depending on the cell context. This means that the same transcription factor can have multiple roles in multiple cell types at different stages of development. Transcription factors, in driving cells down a particular lineage, repress alternative cell fates at the same time.

Transcription factors in the development of dopamine neurons

Mesencephalic DANs are of tremendous research interest because the loss of these neurons in Parkinson's disease contributes to the debilitating effects on control of movement. Experiments in both animals and humans suggest that replacing these neurons not only alleviate but also reverse the physical deterioration significantly (Bjorklund and Kordower, 2013; Kim et al., 2002). As such, there are important clinical applications for an unlimited source of midbrain DANs, stem cell-derived neurons being the forerunner in the search for such sources.

DANs in the central nervous system (CNS) are localized in several cell groups, including those which lie in the midbrain, the hypothalamus and the olfactory bulb regions. This thesis focuses on the midbrain DANs that lies in the substantia nigra and ventral tegmental area (groups A8-A10). Developmental studies conducted in the chick and the mouse models revealed a number of transcriptions factors that are vital to the specification of midbrain DANs at various stages (see Figure 2 for details) – including *Lmx1a, Lmx1b, Foxa2 (Hnf3b), Otx2, En1/2, Pitx3* and *Nurr1*. Other than the expression of these transcription factors, differentiated DAN are identified in cultures

as cells that express the enzymes tyrosine kinase (TH), aldehyde dehydrogenase (Aldh), aromatic L-amino acid decarboxylase (AADC), as well as the dopamine transporter (DAT), vesicular monoamine transporter (VMAT) and/or tyrosine kinase receptor (RET). It is noteworthy that TH, other than being essential for the synthesis of dopamine in DANs, it is also expressed by other neural subtypes such as the NANs. The gold standard in proving their identity therefore lies in transplanting *in-vitro* derived DANs into animals and showing that they are able to integrate and to reverse motor deficits.

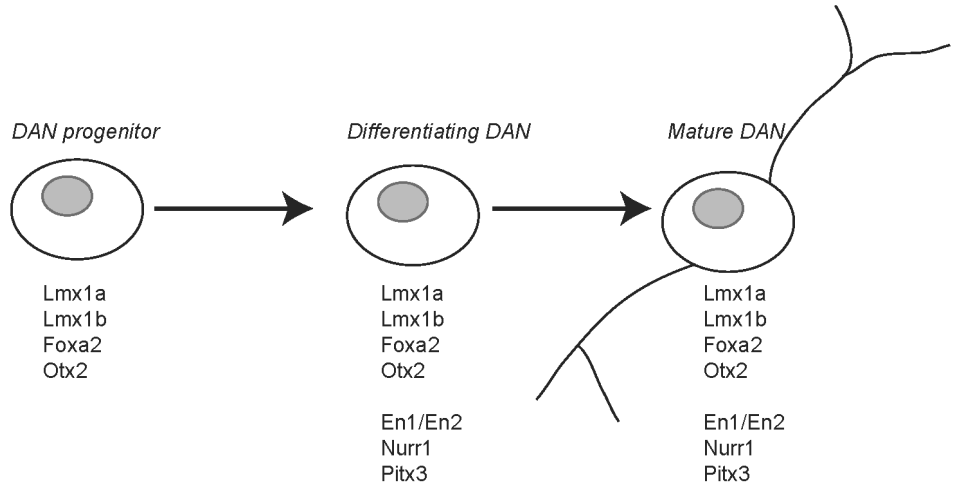

Figure 2: Key transcription factors in dopamine neuron (DAN) development in the mouse and their timing in expression. Lmx1a is expressed early in the DAN progenitor and continues to be expressed in the differentiated mature DAN.

In Paper II, we described the application of a combination of appropriate extrinsic factors – Shh and FGF8, together with the forced expression of Lmx1a in Nestin-positive mouse ESC-derived neural progenitors to generate tyrosine kinase (TH)-positive DAN at a highly efficient rate. The idea of using transcription factors to drive cell fate specification from stem cells is not new. Prior to our attempt at using Lmx1a, Nurr1 and/or Pitx3 overexpression to drive DAN synthesis were studied by numerous others. It is not hard to see why these two transcription factors were favoured over the rest - Nurr1 knockout mice display one of the most severe phenotype in terms of the loss of mesencephalic DANs (Wallen et al., 1999; Zetterstrom et al., 1997). The loss of Pitx3, on the other hand, specifically affects the subset of dopamine neurons that are most vulnerable in Parkinson's disease – namely the A9 group in the substantia nigra (Hwang et al., 2003). Forced expression of Nurr1 or Pitx3, either singly or in combination using viral vectors in neural precursor cells did not induce their

differentiation into DAN in some instances (Sakurada et al., 1999; Sonntag et al., 2004). Others enjoyed greater success by using embryonic neural precursor cells (Kim et al., 2003), or by selecting for "best-performing" mouse ESC-derived clones that were genetically manipulated to overexpress Nurr1, boosting the percentage of TH-positive neurons amongst Tubb3-expressing neurons from 5-10% to up to 90% (Chung et al., 2005; Chung et al., 2002; Kim et al., 2006). When Nurr1 is over-expressed together with the pro-neural gene Ngn2 in fetal mouse ventral midbrain progenitors, *Ngn2* expectedly takes on the role of driving neuronal differentiation, while the addition of Nurr1 leads to the acquisition of dopaminergic markers (Andersson et al., 2007). Overexpressing Pitx3 in mouse ESCs enhanced the proportion of aldehyde dehydrogenase 2 (AHD2)-expressing TH-positive DANs but not the total number of DANs (Chung et al., 2005). AHD2 is mostly expressed in A9 DAN and less so in A8 and A10. An important observation from such studies is the fact that Nurr1 and Pitx3 have specific and unique effects when present either by itself (Nurr1 upregulates TH; Pitx3 upregulates AHD2) or in combination (Nurr1 and Pitx3 upregulates DAT) (Martinat et al., 2006). In summary, Nurr1 or Pitx3 overexpression drives the gene expression of their target genes (TH, RET by Nurr1, AHD2 by Pitx3) hence inducing a "dopaminergic phenotype" without direct effects on neurogenesis or other dopamine transcription factors.

To engineer DAN from stem cells efficiently, we need to consider the individual actions of each intrinsic determinant. Since Nurr1 and Pitx3 start to be expressed only in the early postmitotic DAN between E10.5 and E11.5, we sought to achieve a better induction with some of the earlier expressed dopamine transcription factors such as Lmx1a. Whether or not some of these engineered DANs acquire the complete dopaminergic phenotype requires further study; although I will argue that such genetically engineered neurons are good enough as long as they can perform a functional rescue in animal models of PD, as shown in some studies (Martinat et al., 2006).

Lmx1a is detected by E9 in the embryonic mouse brain (Figure 2). Lmx1a was described to be a key determinant of DAN because it not only induces ectopic DANs when misexpressed in the chick midbrain, it also induced dopamine neuronal generation from mouse and human ESCs put in a permissive environment of Shh and FGF8 (Andersson et al., 2006). The examples described earlier on Nurr1 overexpression studies highlights the importance of the context of cell differentiation - As shown by Nefzger and colleagues, even when Lmx1a is expressed in neural

progenitors, it does not result in the dopaminergic lineage by default (Nefzger et al., 2012). In addition, when Roybon and colleagues overexpressed Lmx1a, Msx1, Ngn2 and Pitx3 either individually or in combination in rat and human neural progenitor cells, they failed to enhance the number of DANs in the culture (Roybon et al., 2008). We need to keep in mind that Lmx1a has specification roles in other cell types such as those in the inner ear and in the roof plate (Koo et al., 2009; Millonig et al., 2000). The same is true for a few of the other key players in dopamine neuron specification, such as Pitx3 in muscle and lens development (Coulon et al., 2007; Medina-Martinez et al., 2009). What is unique about our approach is that Lmx1a turns on only in Nestin-positive neural progenitor cells (and not in pluripotent stem cells, for example) that are exposed to the "midbrain environment". I will elaborate on this in the results section for Paper II.

Phox2a and Phox2b in the development of motor neurons and noradrenergic neurons

Noradrenergic neurons (NANs) in the locus coeruleus (LC) is the major source of norepinephrine to extensive areas in the central nervous system including the cortex and the hippocampus. It has becoming increasingly evident that in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease noradrenaline content in terminal regions is reduced, with an even greater degree of noradrenergic cell loss than that in the site that causes the typical clinical features (i.e. substantia nigra and nucleus basalis) (Bertrand et al., 1997; Cash et al., 1987; Chan-Palay and Asan, 1989; Lyness et al., 2003; Patt and Gerhard, 1993; Tomlinson et al., 1981; Zarow et al., 2003; Zweig et al., 1993). Diminished NANs in these patients contributes to dementia and affective mood disorders such as depression (Marien et al., 2004; Zweig et al., 1993). In the neurodevelopmental disorder Rett syndrome, MECP2 deficiency in the locus coeruleus is suggested to be the greatest contributor to debilitating defects such as respiration and cognition (Amir et al., 1999; Taneja et al., 2009).

Visceral motor neurons (vMN) in the hindbrain innervate the branchial muscles, cardiac muscles and smooth muscles of the viscera. A special group of vMN is found in the oculomotor nuclei in the midbrain. The oculomotor (nIII) and trochlear (nIV) nuclei in the midbrain, together with the abducent nuclei in the hindbrain supply the extraocular muscles of the eyes. Motor neurons (MN) are lost in a myraid of motor neuron diseases such as amyotrophic lateral sclerosis, primary lateral sclerosis, etc. Developmental disorders that are associated with defects in visceral motor neuronal development exist but are rare. These include the Pierre-Robin syndrome, Moebius

and some types of autism (Abadie et al., 2002; Bonanni and Guerrini, 1999; Rodier et al., 1996). Mutations in the PHOX2A gene have been described to cause congenital fibrosis of the extraocular muscles in humans (Nakano et al., 2001).

Despite being involved in some of the most common neurodegenerative diseases, there are no reported protocols to efficiently derive central NANs from ESCs. In-vivo, the LC and oculomotor and trochlear nuclei are sparse and scattered, making ES-derived neurons an attractive alternative to dissected material for study.

The Phox2 transcription factors are expressed in largely overlapping regions in the central nervous system, although differing in their onset and persistence of expression (Pattyn et al., 1997; Tiveron et al., 1996). Targeted deletions of Phox2a and Phox2b have revealed roles not only in the specification of the noradrenergic and visceral motor neuronal cell fate but also requirements in maintaining the phenotype in adult neurons (Coppola et al., 2010; Morin et al., 1997; Pattyn, 2000). In cell lines, both Phox2a and Phox2b can bind to the promoter of the norepinephrine-synthesising enzyme dopamine beta-hydroxylase (Dbh) and stimulate Dbh activity (Kim et al., 1998; Swanson et al., 1997; Yang et al., 1998). Phox2a can even induce noradrenergic synthesis in ectopic locations in the zebrafish (Guo et al., 1999). Exogenous Phox2a in the chick midbrain is able to not only generate the oculomotor nuclei, but also guide motor neurons in ectopic positions to their correct spatial positions (Hasan et al., 2010).

Mammalian homologues Phox2a and Phox2b have identical DNA-binding homeodomains but distinct C-terminal domains (Pattyn et al., 1997). The distinction in their functions seems to be related to the order in their onset of expression in the developing nervous system. In NANs in the hindbrain, Phox2a is expressed in progenitors and turns on Phox2b in cells that have just exited the cell cycle. Phox2b then diminishes by E11.5 while Phox2a stays on for the lifetime of the animal. The oculomotor and trochlear nuclei in the midbrain also expresses Phox2a by E9.0, before Phox2b appears at E10.5 in differentiating neurons. Phox2a-/- mice lack the LC and the oculomotor and trochlear nuclei, amidst other missing structures (Morin et al., 1997; Pattyn et al., 1997). Phox2a is however dispensable for hindbrain visceral motor neuron synthesis (Pattyn et al., 1997). In hindbrain MN, Phox2b comes on first in dividing progenitors then turns on Phox2a in postmitotic neurons. In Phox2b mutants, hindbrain visceral MN are absent and the LC does not develop properly (Pattyn et al., 2000). In fact, all neuronal structures that express Phox2b are affected except for the nIII and nIV neurons, suggesting a dominant effect of Phox2b in regulating neuronal function (Pattyn, 2000; Pattyn et al., 2000; Pattyn et al., 1999). The predominant effect

of Phox2b, as well as the unique requirement for Phox2a in the oculomotor and trochlear nuclei are further shown in gene replacement experiments whereby the coding regions of Phox2a are swopped for Phox2b, and vice versa (Coppola et al., 2005). Phox2b is able to replace Phox2a in specifying NANs, but not that of midbrain motor nuclei while Phox2a is unable to take over the requirements of Phox2b in specifying noradrenergic and hindbrain visceral motor neurons.

In the developing noradrenergic neuronal precursor, Mash1 switches on Phox2a, which goes on to turn on Phox2b (Hirsch et al., 1998; Lo et al., 1998). Mash1, however, is down-regulated before Dbh comes on. In view of the importance of Phox2 proteins in driving the noradrenergic and motor neuronal fate, we extended the same strategy in Paper II to derive central NANs and midbrain MNs from ESCs.

1.4 Challenges in guiding cell fate decisions and practical methods to overcome them.

ESC-derived cultures are often non-homogenous. Contaminating proliferative cells left in culture, even in minute amounts can contribute to disastrous overgrowths in grafts or assays. Although a variety of cell types have been reported to be successfully derived from ESCs, we still need to improve the efficiency of derivation and increase the purity of cells obtained.

Differentiation efficiency

To increase differentiation efficiency, we can start the differentiation from lineage-restricted progenitors instead of pluripotent ESCs. This strategy is used by salamanders in the regeneration of limb tissues and neurons and may serve as an important point to consider in stem cell engineering. When a limb is amputated from the salamander, it triggers the formation of a layer of progenitor cells known as the blastema. Contrary to belief that the blastema consists of homogenous cells that are de-differentiated to gain pluripotency, it was shown in the axolotl that blastema cells retained memory of their former self and were restricted to regenerating cells of the same lineages (Kragl et al., 2009). In the red spotted newt, ablation of DANs using 6-hydroxydopamine triggers dopamine neurogenesis in the normally quiescent ependymoglia cells (Berg et al., 2010; Parish et al., 2007). In line with the same principle stated above, Paper I describes the creation of mesendodermal progenitor cells

(MPCs) from ESCs by Wnt activation, which can give rise to cells of the cardiac, endothelial, osteogenic and chondrogenic lineages more efficiently that the primitive wild-type ESCs.

Methods for selection of desired cell types

One of the ways to overcome low differentiation efficiency is to purify for the desired cell type. The success of cell sorting depends on the availability of cell-type specific surface markers that can be picked up by primary antibodies, and subsequently secondary antibodies engineered to be picked up by various technologies including flow cytometry and magnetic sorting. ESCs display an advantage over other cell types due to the ease of genetic manipulation. For instance, we can generate ESCs that express a fluorescent marker under a lineage-specific driver, and select for the desired cell types using fluorescence-activated cell sorting (Ganat et al., 2012; Hedlund et al., 2008). For cell types that are sensitive to mechanical stress from cell sorting, like neurons, magnetic sorting presents an alternative that may be gentler on the cells with shorter sorting procedures. Magnetically-labelled cells can continue to be grown on tissue culture dishes or transplanted into animals as the magnets are biodegradable. In Papers II and III, we used the MACS® technology to purify for polysialylated-neural cell adhesion molecule (PSA-NCAM) positive neurons. When ESC-derived DANs were sorted this way, they eliminated overgrowth observed on grafts conducted in the same way with cells that were not sorted (Friling et al., 2009b).

Ethical issues

Embryonic stem cells, in particular human ESCs are less acceptable on ethical grounds since they are first derived from blastocysts, the starting point of a human life. Most of the research conducted on human ESCs is however, based on human ESC lines well-established in culture (and not from numerous other human blastocysts). There are also ethical implications in using ESC-derived cells for cell replacement therapy since the ESC was established from another human being. The breakthrough in iPSCs is hence timely to provide a bridge for stem cell research to achieve clinical relevance in overcoming both the ethical issues as well as practical issues such as immune reactions triggered by the grafts.

1.5 A short note on transdifferentiation

There is another route to regenerating lost cells in a dish other than from pluripotent stem cells - in transdifferentiation, we see the direct conversion of one cell type to another specialized cell type by forced expression of appropriate transcription factors. The concept of transdifferentiation is not completely new but it gained momentum following the reprogramming of fibroblasts into iPSCs with merely four transcription factors, which essentially shows that the differentiated cell state is not irreversible (Takahashi and Yamanaka, 2006).

Waddington's epigenetic landscape model perhaps best illustrates the developmental potential of cells (Slack, 2002; Waddington, 1957). The differentiating cell is visualised to be a ball rolling on a surface of hills and valleys and a pluripotent cell with multiple potential lies right on top of a hill. As each cell differentiates and matures, it rolls down the hill, comes across several possible pathways, makes its choice and eventually sits inside one of the valleys. To induce pluripotency will mean taking a somatic cell from a valley to right on top of the hill. This requires more effort than for example, moving the cell across the bump to another valley beside it (analogous to transdifferentiation). Some interesting examples involving clinically relevant cell types include the reprogramming of pancreatic exocrine cells into their closely-related beta cells that secrete insulin (insulin-producing cells appear after 3 days) using only three transcription factors *Pdx1*, Ngn3 and *Mafa* (Zhou et al., 2008); Reprogramming fibroblasts to neurons by *Ascl1*, *Brn2* and *Myt1l* (Pfisterer et al., 2011b; Vierbuchen et al., 2010); Direct conversion of human fibroblasts to DANs (Pfisterer et al., 2011a); Transforming adult fibroblasts to cardiomyocytes by *Gata4*, *Mef2c* and *Tbx5* (Ieda et al., 2010).

Transdifferentiated cells, if the correct combination of transcription factors can be found, is an attractive option that requires shorter time, less costs and poses lower risks for tumour formation after transplantation. The use of either reprogramming techniques depends on the underlying goal – for instance, we can only model early developmental processes in the diseased state (with possibilities of scaling up) or correct genetic mutations using patient-specific iPSCs. If the goal is for cell replacement therapy, then obtaining iPSCs from patients for differentiation, or direct transdifferentiation of easily accessible somatic cells (such as fibroblasts) are viable options. For example, *in-situ* transdifferentiation from astrocytes to dopamine neurons for Parkinson's disease is another ingenious way to overcome problems associated with the use of stem cells (Torper et al., 2013).

2 AIMS

We aim to characterise and develop methods to enhance differentiation efficiency of ESCs by integrating relevant extrinsic and intrinsic signals, specifically

1. To investigate the long-term effects of activation of the Wnt pathway in ESCs differentiation using a chemical inhibitor. (Paper I)

2. To achieve efficient generation of clinically-relevant neuronal subtypes from ESCs (dopamine, motor and serotonergic neurons) by forcing the timed expression of transcription factors key to their development, in the presence of a permissive environment. Such ESC-derived neurons can then be used for the discovery of new markers as well as to identify new pathways key to their development (Paper II).

3. To build on the general principle in Paper II and expand the array of clinically-relevant neurons to include noradrenergic neurons and midbrain-specific motor neurons. We also sought to test the functionality of such ESC-derived neurons in simple drug assays. (Paper III)

3 RESULTS AND DISCUSSION

In this section, I will provide a summary of the results and methods in each paper, highlight some of the key findings, then discuss each paper in line with the theme of the thesis.

Paper I: Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation.

The objective of this study was to investigate the long-term effects of Wnt activation on ESCs pluripotency. To activate the Wnt pathway in ESC, we treated mouse and human ESCs with either home-made Wnt3A conditioned media, recombinant Wnt3A protein, 6-bromoindirubin-3'-oxime (BIO) or a selective inhibitor of glycogen synthase kinase 3β (i-GSK3β), in the presence of LIF. All of these resulted in robust activation of the pathway as indicated by luciferase reporter assays and immunocytochemistry for the active form of beta-catenin in the cells. We compared the effects of i-GSK3β and Wnt3A conditioned media and found that both of them acted similarly to: induce a panel of meso/endodermal markers (*Foxa2, Gata4, Sox17*); down-regulate a panel of ectodermal markers (*Nestin, Pax6, Sox4*) (by quantitative polymerase chain reaction (qPCR) and/or immunocytochemistry); give rise to a greater propensity to differentiate to endothelial sprouts. The Wnt3A conditioned media was an important control for the chemical inhibitor we are using because it showed that the effects observed were due specifically to activation of the Wnt pathway. The up-regulation of markers appeared by day 10 of treatment, and continued to rise when analysed three weeks later. The Wnt-treated mouse ESCs remained proliferative in ES media for up to a year although cells appeared flatter than wild-type ESCs. These ESCs also stain positive for alkaline phosphatase, albeit with lighter staining intensity than wild-type E14.

We hypothesized that the full extent of up-regulation of markers and their enhanced potential to differentiate to the meso/endodermal lineage were masked by heterogeneity in the treated cultures. Hence, to further improve their differentiation efficiency, we isolated single-cell clones from long-term i-GSK3β treated mouse ESCs. i-GSK3β provided a chemically defined alternative to Wnt conditioned media and is more suited to good manufacturing practice (GMP) protocols. Of these, a few clones which displayed slight differences in appearances were picked for further analysis.

Each of these clones displayed a unique pattern of gene expression in terms of the extent of up-regulation of mesendodermal markers. This was similarly reflected in their differentiation potentials. All clones showed more efficient differentiation in assays for the endothelial, cardiac, osteogenic and chondrogenic lineages, while each clone possesses a unique propensity for a certain lineage. For instance, clone 23 with the highest fold change in Gata4 and Tbx5 also produced more embryoid bodies (EBs) with cardiac potential (65% in clone 23 versus 15% in wild-type ESC). Such EBs with cardiac potential were defined as EBs that were "beating" in culture. The "mesendodermal progenitor clones" (MPCs) maintained a stable phenotype in culture for more than a year, and the phenotype was reversible upon i-GSK3β withdrawal (recovery MPCs).

Our study provides a way to use a chemically defined inhibitor (i-GSK3β) to generate renewable mesendodermal progenitor lines from mouse ESC, hence paving the way to large-scale, standardised and reproducible production of desired cell types in therapy.

Prior to our study, a group reported the use of another GSK-3 inhibitor, 6-bromoindirubin-3'-oxime (BIO), to maintain the pluripotency of both mouse and human ESCs (Sato et al., 2004). In the absence of LIF, BIO-treated mouse and human ESC maintained the appearance of tight and compact colonies, Oct3/4 expression as well as the ability to form teratomas and to differentiate into multiple lineages *in-vitro* (Sato et al., 2004). In 2011, ten Berge and colleagues reported the conversion of mouse ESCs to epiblast stem cells with a Wnt antagonist and its rescue with Wnt3a protein and LIF (ten Berge et al., 2011). Wray et. al. also described the requirement for beta-catenin to relieve Tcf3-mediated repression on pluripotency factors in mouse ESCs (Wray et al., 2011).

Wnt activity in the pre- and post-implantation embryo has been visualised in mouse blastocysts with the $Axin2^{LacZ/+}$ reporter. Notably, Wnt is active in E3.5 and E4.5 ICM disappears from the implanted E5.5 to E6.0 embryo before re-appearing again at E6.5 in the posterior end of the embryo, coinciding with the primitive streak and newly derived mesodermal cells (ten Berge et al., 2011),. In cultured EBs with the same reporter, adding Wnt3a proteins served to polarize the EBs in a similar fashion as seen in post-implantation embryos (ten Berge et al., 2008).

What is notable about the studies on using Wnt activation to maintain ESC pluripotency is the relatively shorter period of time the ESCs remained in culture (about 3 passages), compared to our study in which cells were treated for at least 10 days

(around 5 passages) and Davidson et. al's report in which they tracked the effects of Wnt on human ESCs for up to 8 weeks (Davidson et al., 2012). If we compare MPCs with epiblast stem cells (EpiSCs) first reported only in 2007, there are two striking similarities - 1. Mesendodermal markers: EpiSCs were found to co-express mesendodermal markers including Brachyury, Gsc and Sox17 despite expressing similar levels of Oct4, Sox2 and Nanog as ESCs (Bernemann et al., 2011; Tesar et al., 2007). 2. Appearance: EpiSCs grow as flat, compact colonies which resemble MPCs (Brons et al., 2007).

Another key difference between studies is the different pathways that were activated/inhibited together with Wnt activation. Ying et. al. dissected the signalling requirements for ESC pluripotency and their observations explain in part why our results differ (Ying et al., 2008). In simple terms, LIF(Stat3)/serum(BMP) act downstream of ERK/FGF. ERK/FGF stimulates differentiation, and LIF/BMP acts by inhibiting such stimulation. ERK/FGF inhibition alone limits differentiation with "occasional neural rosettes". A combination of ERK/FGF inhibition and Wnt activation (by GSK-3 inhibition) is sufficient to maintain ESC pluripotency, independent of Stat3-mediated self-renewal.

In a recent paper, Habib et. al. applied local Wnt signals (in the presence of LIF) to mouse ESCs using Wnt3a immobilized on beads (Habib et al., 2013). They described how a gradient in Wnt signalling resulted in the Wnt-proximal daughter cell expressing higher levels of pluripotency associated genes and the Wnt-distal daughter cell expressing differentiation markers with the hallmarks of EpiSCs.

Hence, it is possible that in our study Wnt activation plus LIF supported pluripotency to ESCs initially. With long-term in vitro culture, LIF-mediated inhibition of differentiation signals started to break down and some ESCs drifted towards a less primitive state that was "EpiSCs-like". This state was then stabilised by Wnt activation. With continued selection and the pressure to move down the differentiation path, most of the ESCs eventually adopt the "EpiSCs-like" state and facilitated our derivation of MPCs. Unfortunately, due to limited analysis of MPCs, I can only speculate on the possibilities at this juncture.

Paper II: Transcription factor-induced lineage selection of stem-cell-derived neural progenitor cells.

In this study we developed a general strategy for efficient generation of clinically-relevant neuronal subtypes from mouse ESCs (dopamine neurons, visceral

motor neurons, somatic motor neurons, serotonin neurons) by forcing the timed expression of transcription factors key to their development (Lmx1a, Phox2b, Olig2, Nkx2.2) in the presence of appropriate extrinsic signals (sonic hedgehog (Shh) , fibroblast growth factor 8 (FGF8), retinoic acid (RA)). We made stable ESC lines expressing transcription factors under the control of the Nestin enhancer (NesE-Lmx1a, NesE-Phox2b, NesE-Olig2, NesE-Nkx2.2) and differentiated them as a monolayer in the presence of various growth factors. The differentiation efficiency was analysed by co-expression of key markers by immunocytochemistry as well as cell counts. After purifying the differentiated ESC cultures for neurons by magnetic sorting (for PSA-NCAM), we subjected the populations of neurons to genome-wide expression analysis (Affymetrix arrays) and showed that most of the genes are indeed expressed in their *in-vivo* counterparts by *in-situ* hybridization in the mouse embryonic brain. This allowed us to not only identify new markers, but also to uncover previously unknown features such as the similarity between dopamine and serotonin neurons (by hierarchical clustering), and the importance of the insulin growth factor signalling pathway to dopamine neuron generation. We then showed that ESC-derived neural progenitors that are rendered "noncompetent" to differentiate to dopamine neurons (in the presence of retinoic acid) can be reprogrammed by a combination of transcription factors (lentiviral-driven En1, Otx2). This method can even be extended to noncompetent human forebrain-derived neuronal progenitor cells, hence presenting an effective method to overcome limitations in deriving clinically-relevant cell types.

Dopamine neurons derived from NesE-Lmx1a ESCs have been shown to successfully engraft and reinnervate 6-hydroxydopamine lesioned rat striatum (Friling et al., 2009b). Following our study, another group has shown that our strategy (of overexpressing Lmx1a in Nestin-positive progenitors) also works in human iPSCs to increase the efficiency of dopamine neuron derivation (Sanchez-Danes et al., 2012a).

There were a few key factors that contributed to the success in achieving high differentiation efficiencies of various neuronal subtypes from mouse ESCs – Choice of transcription factor in relevance to role in developmental cascade; Creating a permissive environment; Timing of overexpression.

A number of transcriptions factors are known to be vital to the specification of dopamine neurons at various stages – including *Lmx1a, Lmx1b, Foxa2, Otx2, En1/2, Pitx3* and *Nurr1* (Figure 2). As previously discussed in the introduction section, Nurr1 and Pitx3 has garnered more interest than all the other transcription factors in the hunt for transcription factors to induce dopamine neuron differentiation from stem cells. It

seems surprising at first glance that a transcription factor such as Lmx1a, with its mild effects on dopamine neuron development when knocked out in the mice, turns out to be one of the most robust candidate in driving dopamine neuron synthesis from ESCs (Deng et al., 2011). Numerous other groups have reproduced the efficient generation of midbrain dopamine neurons by overexpressing Lmx1a (Sanchez-Danes et al., 2012a).

Lmx1a is one of the few transcription factors (*Lmx1b, Foxa2, En1, Otx2*) that is expressed earliest in dopamine neuronal progenitors (E9). In the developing central nervous system (CNS), Lmx1a is an important intrinsic determinant not only in developing dopamine neurons but also in the roof plate (Andersson et al., 2006; Millonig et al., 2000). In ventral CNS, however, unlike most of the other key DAN transcription factors, Lmx1a is specifically expressed only in dopamine neurons. Hence, in the presence of Shh (to ventralise the culture) and FGF8 (to provide the midbrain positional information), Lmx1a expression in Nestin-positive progenitors successfully directs their differentiation to dopamine neurons. The importance of appropriate extrinsic factors is also highlighted in the study by Wagner and colleagues – Nurr1 overexpression induced the midbrain dopamine phenotype in C17.2 neural stem cells only in the presence of signals from the ventral mesencephalon (Wagner et al., 1999).

Paper III: Transcription factor-induced lineage programming of noradrenaline and visceral motor neurons from embryonic stem cells.

This study builds on the general principle for the generation of specific neuronal cell types from ESCs that we developed in Paper II. We applied this strategy with the goal of generating noradrenaline neurons (NAN) from mouse ESCs. Mouse ESC-derived neural progenitors were found to give rise to the highest numbers of NANs in the presence of bone morphogenetic protein 7 (BMP7) and fibroblast growth factor (FGF8). Based on the developmental requirements of Phox2 transcription factors in NAN specification, we forced the expression of Phox2a and Phox2b in ESC-derived neural progenitors to improve the efficiency of NAN synthesis. This was done by constructing stable mouse ESC lines expressing Phox2a or Phox2b under the control of the Nestin enhancer (NesE-Phox2a or NesE-Phox2b). Phox2 transcription factors are also key to the specification of visceral motor neurons (vMN), which, as shown in Paper II, requires Shh and FGF8. NesE-Phox2b differentiated in the presence of BMP7 and FGF8 further improved the efficiency by which NAN-like neurons are derived, based on marker analysis by quantitative polymerase chain reaction (qPCR),

immunocytochemistry and cell counts. NesE-Phox2a, on the other hand, only improved vMN generation in the presence of Shh and FGF8. vMN synthesis can similarly be improved using the NesE-Phox2b line (shown in this paper and Paper II).

After purifying the differentiated ESC cultures for neurons by magnetic sorting (for PSA-NCAM), we subjected the ESC-derived NAN and vMN to genome-wide expression analysis (Affymetrix arrays). The gene lists allow novel NAN markers and midbrain-specific vMN markers to be identified, some of which are verified by *in-situ* hybridization studies in the mouse embryonic brain. The practical application of these ESC-derived neurons were tested in a simple high-throughput imaging assay to show that they can be used *in-vitro* to screen for drug specificity and toxicity. ASP+ (4-(4-(dimethylamino)styryl)-N-methylpyridinium) is a fluorescent substrate that mimics neurotransmitter binding. When drugs that inhibit binding and uptake of various neurotransmitters are added to NAN- and vMN-enriched cultures, they reduced ASP+ binding according to the neuronal subtypes present in the cultures. As a further step to test this method in generating neuronal cultures of clinical relevance, we overexpressed Phox2a or Phox2b in human ESCs and improved the efficiency by which NANs and vMN are derived.

With this paper, we expanded the array of clinically-relevant neurons to include NANs and midbrain vMNs that can be efficiently derived from ESCs. This paper also emphasized the concept of an appropriate permissive environment using the NesE-Phox2b line. By ventralizing or dorsalizing the culture with Shh or BMPs respectively, the same NesE-Phox2b line drives vMN or NAN synthesis respectively. Despite the importance of Phox2a in driving NAN synthesis in vivo, it was shown to be much less effective in vitro, compared to its ability to drive vMN synthesis. Phox2a and Phox2b have identical DNA-binding homeodomains but distinct C-terminal domains (Pattyn et al., 1997). This suggests the possibility of missing cofactors or missing links in our understanding of the transcriptional cascade in the development of NAN. In such case, the list of NAN-enriched genes may provide vital clues. The dominant effect of Phox2b in driving NAN synthesis is also in congruent with results in the reciprocal replacement experiments shown by Coppola et. al. (Coppola et al., 2005).

An additional point made in this paper is the use of ESC-derived neurons as tools in drug assays. Research on neuronal function or neuronal response to drugs has been facilitated for many years by either dissected material from animals or cell lines such as SH-SY5Y or MN9D. We adapted drug assays that use HEK293 cells artificially expressing neurotransmitter transporters/receptors, and replaced the non-

neuronal cells with ESC-derived NAN and vMN (Haunso and Buchanan, 2007; Schwartz et al., 2003; Schwartz et al., 2005). Our assays have shown that subtype-specific response to drugs can be predicted using ESC-derived neurons. Although we did not directly compare non-neuronal cell lines with ESC-derived neurons, it is likely that ESC-derived neurons is more representative of the biological responses seen in *in-vivo* physiological conditions.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The three studies described in this thesis highlights the integration of developmentally appropriate extrinsic and intrinsic factors to build rational approaches in stem cell engineering.

In the first study, we explored the use of a chemically defined inhibitor of GSK-3 to activate the Wnt pathway to construct a renewable source of mesendodermal progenitors. Such strategies may even be coupled with genetically engineered cell lines, such as those described in Paper II and III, to give a further boost to differentiation efficiencies. The successful use of chemically defined Wnt inhibitors also indicates that it is timely to explore the replacement of recombinant proteins with chemically defined agents in stem cell differentiation to minimise variation as well as for the long-term goal of being compatible with good manufacturing practices.

In Papers II and III, we integrated knowledge on developmental requirements of neuronal subtypes to build an approach that can be applied to derive numerous clinically-relevant subtypes. It has been suggested and shown in some cases that the specificity of transcription factors lie in the different partners they bind to in a different cell context. I envision that the differentiation efficiencies may be further improved by the combined overexpression of a few transcription factors. In fact, this was the strategy applied by Nefzger et. al. to further enhance the induction of serotonergic neurons from ESCs by adding Mash1 and Foxa2 to Gata2 (Nefzger et al., 2011).

Although there is cause for concern in using genetically modified cells in therapy, we can minimise risks with careful experimental designs. For instance, in our studies in Paper II and III, the Nestin enhancer is in a “off” state after the cells become post-mitotic and this switches off the exogenous expression of the construct. These studies are simply proof-of-principle research and we should continually work towards improving methods to modify the genome in a safer manner. There are already promising advances in non-viral methods for stable modifications of stem cells, such as the episomal vectors, adenovirus, BacMam, etc (Fontes and Lakshmipathy, 2013).

There must be inherent differences between ESC-derived cells and their respective *bona fida* cells but I will argue that as long as such cells are properly characterized for functionality in the specific application (for example, drug screening, disease modelling, transplantation), it will suffice.

5 ACKNOWLEDGEMENTS

I must say that I really enjoyed myself for the past six years doing Science, and a large part of it is due to the people I met in the journey. I will forever be grateful to my supervisors, **Thomas Perlmann** and **Lawrence Stanton**.

To **Thomas**, you took up the challenge to train me into a scientist despite not ever seeing me hold a pipette or load a gel beforehand. Thank you for your trust in me. It has been an extremely fulfilling six years of work!

To **Larry**, you helped me to initiate first contact with Thomas and gave me your utmost support when I decided to take up the offer to come to the Karolinska Institutet. Thank you for all your time in filling up forms, reports and discussions despite my lack of presence in your lab in Singapore!

To **Lia**, you guided me with much patience and were extremely generous in sharing tips on lab techniques. Thank you for being such a great hands-on mentor!

To **Nigel**, my "D-team" partner. You have been extraordinary! I will miss discussing crazy ideas with you. You will make an amazing researcher! Best of luck in the final lap of your PhD studies!

To people I worked closely with **Banafsheh**, **Lina**, **Eliza**, **Nick**, **Ariadna**, **Maria Papathanou**, **André, Katarina, Hilda**, **Eva**, **CY**, **Michal**, **Stina**, and the inspiring crowd at LICR and CMB **Maria Bergsland**, **Idha**, **Daniel**, **Cécile**, **Susanne**, **Alexandra**, **Gaelle**, **Magnus**, **Inger**, **Soheilla**, **Minerva**, **Julianna**, **Belma**, **Danny**, **Jan**, **Johan**, **Elisabet**, **Zhanna**, **Jose**, **Chris**, **Sanja**, **Qiaolin**, **Zhixiong**, **Stuart, Vilma, Wang Xun**. I am a lucky girl to meet so many kind and helpful people during the most challenging years of my life. Stockholm will always have a special place in my heart. Thank you for the memories.

To the Singaporean/Malaysian/Chinese gang I met in KI, **Michelle**, **Kaier**, **Jimmie**, **Charis**, **Xiaohui**, **Yinghui**, **Sam & Jinny**, **Kian Chye & Huizhen**, **Enghui**, **Samantha**, **Vithia**, **Weng-Onn**, **Yin-Choy**, **Farhana**, **Delina**, **Chek Mei, Li Meng**, **Li Yu**, hanging out with you guys always make me feel at home and for that brief moment

in time, I forget that I was far far away from sunny Singapore… Thank you for bearing with my occasional rants and keeping my tummy satisfied with Asian food!

To my friends and colleagues in Genome Institute of Singapore, **Sarah**, **Linda**, **Irene**, **Akshay**, **Felicia**, **Priya**, **Jiaxuan**, **Shi Yan**, **Kee Yew**, **Siaw Wei**, **Rani**, **Wan Qing**, **Gireesh**, **Nani**, **Boon Seng**, **Swea Ling**, **Roy**, **Tapan**, **Rory**, **Christina**, **Hong Bing**. I had to trouble many of you as I travelled back and forth. Thank you for always lending me a helping hand! I really appreciate it!

To my mentor Professor **Sim Meng Kwoon**, you planted the seed of passion for science in me. Thank you for your kind patience in guiding a Pharmacy undergraduate in her first lab-based research project and your sincere career guidance. To Professor **Chan Sui Yung,** you went the extra mile to help me in my application to A*STAR. Thank you for everything!

To my loved ones, **my parents**, **my brother Jensen** and **my husband Zyayn**. Thank you for your endless support and for believing in me (^.^)

6 REFERENCES

Abadie, V., Morisseau-Durand, M. P., Beyler, C., Manach, Y., and Couly, G. (2002). Brainstem dysfunction: a possible neuroembryological pathogenesis of isolated Pierre Robin sequence. Eur J Pediatr *161*, 275-280.

Abassi, Y. A., Xi, B., Li, N., Ouyang, W., Seiler, A., Watzele, M., Kettenhofen, R., Bohlen, H., Ehlich, A., Kolossov, E.*, et al.* (2012). Dynamic monitoring of beating periodicity of stem cell-derived cardiomyocytes as a predictive tool for preclinical safety assessment. Br J Pharmacol *165*, 1424-1441.

Alberts, B. (2008). Molecular biology of the cell, 5th edn (New York ; Abingdon: Garland Science).

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nat Genet *23*, 185-188.

Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T., and Ericson, J. (2006). Identification of intrinsic determinants of midbrain dopamine neurons. Cell *124*, 393-405.

Andersson, E. K., Irvin, D. K., Ahlsio, J., and Parmar, M. (2007). Ngn2 and Nurr1 act in synergy to induce midbrain dopaminergic neurons from expanded neural stem and progenitor cells. Exp Cell Res *313*, 1172-1180.

Andersson, E. R., Prakash, N., Cajanek, L., Minina, E., Bryja, V., Bryjova, L., Yamaguchi, T. P., Hall, A. C., Wurst, W., and Arenas, E. (2008). Wnt5a regulates ventral midbrain morphogenesis and the development of A9-A10 dopaminergic cells in vivo. PLoS ONE *3*, e3517.

Andersson, E. R., Salto, C., Villaescusa, J. C., Cajanek, L., Yang, S., Bryjova, L., Nagy, II, Vainio, S. J., Ramirez, C., Bryja, V., and Arenas, E. (2013). Wnt5a cooperates with canonical Wnts to generate midbrain dopaminergic neurons in vivo and in stem cells. Proc Natl Acad Sci U S A *110*, E602-610.

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T., and Yamanaka, S. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science *321*, 699-702.

Aubert, J., Dunstan, H., Chambers, I., and Smith, A. (2002). Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. Nat Biotechnol *20*, 1240-1245.

Bakre, M. M., Hoi, A., Mong, J. C. Y., Koh, Y. Y., Wong, K. Y., and Stanton, L. W. (2007). Generation of multipotential mesendodermal progenitors from mouse embryonic stem cells via sustained Wnt pathway activation. J Biol Chem *282*, 31703-31712.

Bar-Nur, O., Russ, H. A., Efrat, S., and Benvenisty, N. (2011). Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell *9*, 17-23.

Barker, R. A., Barrett, J., Mason, S. L., and Bjorklund, A. (2013). Fetal dopaminergic transplantation trials and the future of neural grafting in Parkinson's disease. Lancet Neurol *12*, 84-91.

Berg, D. A., Kirkham, M., Beljajeva, A., Knapp, D., Habermann, B., Ryge, J., Tanaka, E. M., and Simon, A. (2010). Efficient regeneration by activation of neurogenesis in homeostatically quiescent regions of the adult vertebrate brain. Development *137*, 4127-4134.

Bernemann, C., Greber, B., Ko, K., Sterneckert, J., Han, D. W., Arauzo-Bravo, M. J., and Scholer, H. R. (2011). Distinct developmental ground states of epiblast stem cell lines determine different pluripotency features. STEM CELLS *29*, 1496-1503.

Bertrand, E., Lechowicz, W., Szpak, G. M., and Dymecki, J. (1997). Qualitative and quantitative analysis of locus coeruleus neurons in Parkinson's disease. Folia Neuropathol *35*, 80-86.

Bjorklund, A., and Kordower, J. H. (2013). Cell therapy for Parkinson's disease: what next? Mov Disord *28*, 110-115.

Bonanni, P., and Guerrini, R. (1999). Segmental facial myoclonus in moebius syndrome. Mov Disord *14*, 1021-1024.
Briscoe, J., and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. Curr Opin Neurobiol *11*, 43-49.
Briscoe, J., Pierani, A., Jessell, T. M., and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell *101*, 435-445.
Brons, I. G. M., Smithers, L. E., Trotter, M. W. B., Rugg-Gunn, P., Sun, B., Chuva De Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A., and Vallier, L. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. Nature *448*, 191-195.
Cadigan, K. M., and Waterman, M. L. (2012). TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb Perspect Biol *4*.
Cajánek, L., Ribeiro, D., Liste, I., Parish, C., Bryja, V., and Arenas, E. (2009). Wnt/beta-Catenin Signaling Blockade Promotes Neuronal Induction and Dopaminergic Differentiation in Embryonic Stem Cells. Stem Cells.
Cash, R., Dennis, T., L'Heureux, R., Raisman, R., Javoy-Agid, F., and Scatton, B. (1987). Parkinson's disease and dementia: norepinephrine and dopamine in locus ceruleus. Neurology *37*, 42-46.
Castelo-Branco, G., Wagner, J., Rodriguez, F. J., Kele, J., Sousa, K., Rawal, N., Pasolli, H. A., Fuchs, E., Kitajewski, J., and Arenas, E. (2003). Differential regulation of midbrain dopaminergic neuron development by Wnt-1, Wnt-3a, and Wnt-5a. Proc Natl Acad Sci U S A *100*, 12747-12752.
Chan-Palay, V., and Asan, E. (1989). Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. J Comp Neurol *287*, 373-392.
Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature *383*, 407-413.
Chin, M. H., Mason, M. J., Xie, W., Volinia, S., Singer, M., Peterson, C., Ambartsumyan, G., Aimiuwu, O., Richter, L., Zhang, J.*, et al.* (2009). Induced pluripotent stem cells and embryonic stem cells are distinguished by gene expression signatures. Cell Stem Cell *5*, 111-123.
Chung, S., Hedlund, E., Hwang, M., Kim, D. W., Shin, B. S., Hwang, D. Y., Kang, U. J., Isacson, O., and Kim, K. S. (2005). The homeodomain transcription factor Pitx3 facilitates differentiation of mouse embryonic stem cells into AHD2-expressing dopaminergic neurons. Mol Cell Neurosci *28*, 241-252.
Chung, S., Sonntag, K. C., Andersson, T., Bjorklund, L. M., Park, J. J., Kim, D. W., Kang, U. J., Isacson, O., and Kim, K. S. (2002). Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. Eur J Neurosci *16*, 1829-1838.
Coppola, E., d'Autréaux, F., Rijli, F. M., and Brunet, J.-F. (2010). Ongoing roles of Phox2 homeodomain transcription factors during neuronal differentiation. Development *137*, 4211-4220.
Coppola, E., Pattyn, A., Guthrie, S. C., Goridis, C., and Studer, M. (2005). Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. EMBO J *24*, 4392-4403.
Coulon, V., L'Honoré, A., Ouimette, J.-F., Dumontier, E., van den Munckhof, P., and Drouin, J. (2007). A muscle-specific promoter directs Pitx3 gene expression in skeletal muscle cells. J Biol Chem *282*, 33192-33200.
Dalgetty, D. M., Medine, C. N., Iredale, J. P., and Hay, D. C. (2009). Progress and future challenges in stem cell-derived liver technologies. Am J Physiol Gastrointest Liver Physiol *297*, G241-248.
Davidson, K. C., Adams, A. M., Goodson, J. M., McDonald, C. E., Potter, J. C., Berndt, J. D., Biechele, T. L., Taylor, R. J., and Moon, R. T. (2012). Wnt/β-catenin signaling promotes differentiation, not self-renewal, of human embryonic stem cells and is repressed by Oct4. Proc Natl Acad Sci USA *109*, 4485-4490.
Del Valle, I., Rudloff, S., Carles, A., Li, Y., Liszewska, E., Vogt, R., and Kemler, R. (2013). E-cadherin is required for the proper activation of the Lifr/Gp130 signaling pathway in mouse embryonic stem cells. Development *140*, 1684-1692.

Delgado, J. P., Parouchev, A., Allain, J. E., Pennarun, G., Gauthier, L. R., Dutrillaux, A. M., Dutrillaux, B., Di Santo, J., Capron, F., Boussin, F. D., and Weber, A. (2005). Long-term controlled immortalization of a primate hepatic progenitor cell line after Simian virus 40 T-Antigen gene transfer. Oncogene *24*, 541-551.
Deng, Q., Andersson, E., Hedlund, E., Alekseenko, Z., Coppola, E., Panman, L., Millonig, J. H., Brunet, J. F., Ericson, J., and Perlmann, T. (2011). Specific and integrated roles of Lmx1a, Lmx1b and Phox2a in ventral midbrain development. Development *138*, 3399-3408.
Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., and Hui, C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development *125*, 2533-2543.
Doble, B. W., and Woodgett, J. R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. J Cell Sci *116*, 1175-1186.
Domcke, S., Sinha, R., Levine, D. A., Sander, C., and Schultz, N. (2013). Evaluating cell lines as tumour models by comparison of genomic profiles. Nat Commun *4*, 2126.
Dupe, V., and Lumsden, A. (2001). Hindbrain patterning involves graded responses to retinoic acid signalling. Development *128*, 2199-2208.
Egawa, N., Kitaoka, S., Tsukita, K., Naitoh, M., Takahashi, K., Yamamoto, T., Adachi, F., Kondo, T., Okita, K., Asaka, I.*, et al.* (2012). Drug screening for ALS using patient-specific induced pluripotent stem cells. Sci Transl Med *4*, 145ra104.
Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell *87*, 661-673.
Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M., and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell *90*, 169-180.
Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.
Feng, B., Jiang, J., Kraus, P., Ng, J. H., Heng, J. C., Chan, Y. S., Yaw, L. P., Zhang, W., Loh, Y. H., Han, J.*, et al.* (2009). Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol *11*, 197-203.
Fontes, A., and Lakshmipathy, U. (2013). Advances in Genetic Modification of Pluripotent Stem Cells. Biotechnol Adv.
Forde, J. E., and Dale, T. C. (2007). Glycogen synthase kinase 3: a key regulator of cellular fate. Cell Mol Life Sci *64*, 1930-1944.
Freed, C. R., Breeze, R. E., Rosenberg, N. L., Schneck, S. A., Kriek, E., Qi, J. X., Lone, T., Zhang, Y. B., Snyder, J. A., Wells, T. H., and et al. (1992). Survival of implanted fetal dopamine cells and neurologic improvement 12 to 46 months after transplantation for Parkinson's disease. N Engl J Med *327*, 1549-1555.
Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W. Y., DuMouchel, W., Kao, R., Dillon, S., Winfield, H., Culver, S., Trojanowski, J. Q.*, et al.* (2001). Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med *344*, 710-719.
Friling, S., Andersson, E., Thompson, L. H., Jonsson, M. E., Hebsgaard, J. B., Nanou, E., Alekseenko, Z., Marklund, U., Kjellander, S., Volakakis, N.*, et al.* (2009a). Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells. Proc Natl Acad Sci U S A *106*, 7613-7618.
Friling, S., Andersson, E., Thompson, L. H., Jönsson, M. E., Hebsgaard, J. B., Nanou, E., Alekseenko, Z., Marklund, U., Kjellander, S., Volakakis, N.*, et al.* (2009b). Efficient production of mesencephalic dopamine neurons by Lmx1a expression in embryonic stem cells. Proc Natl Acad Sci USA *106*, 7613-7618.
Ganat, Y. M., Calder, E. L., Kriks, S., Nelander, J., Tu, E. Y., Jia, F., Battista, D., Harrison, N., Parmar, M., Tomishima, M. J.*, et al.* (2012). Identification of embryonic stem cell-derived midbrain dopaminergic neurons for engraftment. J Clin Invest *122*, 2928-2939.
Gaspard, N., Bouschet, T., Herpoel, A., Naeije, G., van den Ameele, J., and Vanderhaeghen, P. (2009). Generation of cortical neurons from mouse embryonic stem cells. Nat Protoc *4*, 1454-1463.
Gilbert, S. F., Tyler, M. S., and Kozlowski, R. N. (2000). Developmental biology, 6th edn (Sunderland, Mass.Basingstoke: Sinauer Associates; Macmillan).

Glover, J. C., Renaud, J. S., and Rijli, F. M. (2006). Retinoic acid and hindbrain patterning. J Neurobiol *66*, 705-725.
Guo, S., Brush, J., Teraoka, H., Goddard, A., Wilson, S. W., Mullins, M. C., and Rosenthal, A. (1999). Development of noradrenergic neurons in the zebrafish hindbrain requires BMP, FGF8, and the homeodomain protein soulless/Phox2a. Neuron *24*, 555-566.
Habib, S. J., Chen, B. C., Tsai, F. C., Anastassiadis, K., Meyer, T., Betzig, E., and Nusse, R. (2013). A localized Wnt signal orients asymmetric stem cell division in vitro. Science *339*, 1445-1448.
Hagell, P., Schrag, A., Piccini, P., Jahanshahi, M., Brown, R., Rehncrona, S., Widner, H., Brundin, P., Rothwell, J. C., Odin, P.*, et al.* (1999). Sequential bilateral transplantation in Parkinson's disease: effects of the second graft. Brain *122 (Pt 6)*, 1121-1132.
Hanna, J., Wernig, M., Markoulaki, S., Sun, C. W., Meissner, A., Cassady, J. P., Beard, C., Brambrink, T., Wu, L. C., Townes, T. M., and Jaenisch, R. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. Science *318*, 1920-1923.
Hasan, K. B., Agarwala, S., and Ragsdale, C. W. (2010). PHOX2A regulation of oculomotor complex nucleogenesis. Development *137*, 1205-1213.
Haunso, A., and Buchanan, D. (2007). Pharmacological characterization of a fluorescent uptake assay for the noradrenaline transporter. J Biomol Screen *12*, 378-384.
Hedlund, E., Pruszak, J., Lardaro, T., Ludwig, W., Vinuela, A., Kim, K. S., and Isacson, O. (2008). Embryonic stem cell-derived Pitx3-enhanced green fluorescent protein midbrain dopamine neurons survive enrichment by fluorescence-activated cell sorting and function in an animal model of Parkinson's disease. STEM CELLS *26*, 1526-1536.
Heng, J. C., Feng, B., Han, J., Jiang, J., Kraus, P., Ng, J. H., Orlov, Y. L., Huss, M., Yang, L., Lufkin, T.*, et al.* (2010). The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell *6*, 167-174.
Hidalgo-Sanchez, M., Simeone, A., and Alvarado-Mallart, R. M. (1999). Fgf8 and Gbx2 induction concomitant with Otx2 repression is correlated with midbrain-hindbrain fate of caudal prosencephalon. Development *126*, 3191-3203.
Hirsch, M. R., Tiveron, M. C., Guillemot, F., Brunet, J. F., and Goridis, C. (1998). Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. Development *125*, 599-608.
Hwang, D. Y., Ardayfio, P., Kang, U. J., Semina, E. V., and Kim, K. S. (2003). Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. Brain Res Mol Brain Res *114*, 123-131.
Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell *142*, 375-386.
Joksimovic, M., Yun, B. A., Kittappa, R., Anderegg, A. M., Chang, W. W., Taketo, M. M., McKay, R. D., and Awatramani, R. B. (2009). Wnt antagonism of Shh facilitates midbrain floor plate neurogenesis. Nat Neurosci *12*, 125-131.
Kettenhofen, R., and Bohlen, H. (2008). Preclinical assessment of cardiac toxicity. Drug Discov Today *13*, 702-707.
Kim, D. W., Chung, S., Hwang, M., Ferree, A., Tsai, H. C., Park, J. J., Nam, T. S., Kang, U. J., Isacson, O., and Kim, K. S. (2006). Stromal cell-derived inducing activity, Nurr1, and signaling molecules synergistically induce dopaminergic neurons from mouse embryonic stem cells. STEM CELLS *24*, 557-567.
Kim, H. S., Seo, H., Yang, C., Brunet, J. F., and Kim, K. S. (1998). Noradrenergic-specific transcription of the dopamine beta-hydroxylase gene requires synergy of multiple cis-acting elements including at least two Phox2a-binding sites. J Neurosci *18*, 8247-8260.
Kim, J. B., Zaehres, H., Arauzo-Bravo, M. J., and Scholer, H. R. (2009a). Generation of induced pluripotent stem cells from neural stem cells. Nat Protoc *4*, 1464-1470.
Kim, J. B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Arauzo-Bravo, M. J., Ruau, D., Han, D. W., Zenke, M., and Scholer, H. R. (2008). Pluripotent stem cells

induced from adult neural stem cells by reprogramming with two factors. Nature *454*, 646-650.
Kim, J. H., Auerbach, J. M., Rodriguez-Gomez, J. A., Velasco, I., Gavin, D., Lumelsky, N., Lee, S. H., Nguyen, J., Sanchez-Pernaute, R., Bankiewicz, K., and McKay, R. (2002). Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature *418*, 50-56.
Kim, J. Y., Koh, H. C., Lee, J. Y., Chang, M. Y., Kim, Y. C., Chung, H. Y., Son, H., Lee, Y. S., Studer, L., McKay, R., and Lee, S. H. (2003). Dopaminergic neuronal differentiation from rat embryonic neural precursors by Nurr1 overexpression. J Neurochem *85*, 1443-1454.
Kim, K., Zhao, R., Doi, A., Ng, K., Unternaehrer, J., Cahan, P., Huo, H., Loh, Y. H., Aryee, M. J., Lensch, M. W*., et al.* (2011a). Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. Nat Biotechnol *29*, 1117-1119.
Kim, K. Y., Hysolli, E., and Park, I. H. (2011b). Neuronal maturation defect in induced pluripotent stem cells from patients with Rett syndrome. Proc Natl Acad Sci U S A *108*, 14169-14174.
Kim, M., Habiba, A., Doherty, J. M., Mills, J. C., Mercer, R. W., and Huettner, J. E. (2009b). Regulation of mouse embryonic stem cell neural differentiation by retinoic acid. Dev Biol *328*, 456-471.
Kinder, S. J., Tsang, T. E., Wakamiya, M., Sasaki, H., Behringer, R. R., Nagy, A., and Tam, P. P. (2001). The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. Development *128*, 3623-3634.
Kirkeby, A., Grealish, S., Wolf, D. A., Nelander, J., Wood, J., Lundblad, M., Lindvall, O., and Parmar, M. (2012). Generation of regionally specified neural progenitors and functional neurons from human embryonic stem cells under defined conditions. Cell Rep *1*, 703-714.
Kondo, T., Asai, M., Tsukita, K., Kutoku, Y., Ohsawa, Y., Sunada, Y., Imamura, K., Egawa, N., Yahata, N., Okita, K*., et al.* (2013). Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness. Cell Stem Cell *12*, 487-496.
Koo, S. K., Hill, J. K., Hwang, C. H., Lin, Z. S., Millen, K. J., and Wu, D. K. (2009). Lmx1a maintains proper neurogenic, sensory, and non-sensory domains in the mammalian inner ear. Dev Biol *333*, 14-25.
Kragl, M., Knapp, D., Nacu, E., Khattak, S., Maden, M., Epperlein, H. H., and Tanaka, E. M. (2009). Cells keep a memory of their tissue origin during axolotl limb regeneration. Nature *460*, 60-65.
Kraushaar, U., Meyer, T., Hess, D., Gepstein, L., Mummery, C. L., Braam, S. R., and Guenther, E. (2012). Cardiac safety pharmacology: from human ether-a-gogo related gene channel block towards induced pluripotent stem cell based disease models. Expert Opin Drug Saf *11*, 285-298.
Kubo, A., Shinozaki, K., Shannon, J. M., Kouskoff, V., Kennedy, M., Woo, S., Fehling, H. J., and Keller, G. (2004). Development of definitive endoderm from embryonic stem cells in culture. Development *131*, 1651-1662.
Lan, F., Lee, A. S., Liang, P., Sanchez-Freire, V., Nguyen, P. K., Wang, L., Han, L., Yen, M., Wang, Y., Sun, N*., et al.* (2013). Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. Cell Stem Cell *12*, 101-113.
Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. Development *113*, 891-911.
Lee, K. J., Mendelsohn, M., and Jessell, T. M. (1998). Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev *12*, 3394-3407.
Lek, M., Dias, J. M., Marklund, U., Uhde, C. W., Kurdija, S., Lei, Q., Sussel, L., Rubenstein, J. L., Matise, M. P., Arnold, H. H*., et al.* (2010). A homeodomain feedback circuit underlies step-function interpretation of a Shh morphogen gradient during ventral neural patterning. Development *137*, 4051-4060.
Li, X. J., Du, Z. W., Zarnowska, E. D., Pankratz, M., Hansen, L. O., Pearce, R. A., and Zhang, S. C. (2005). Specification of motoneurons from human embryonic stem cells. Nat Biotechnol *23*, 215-221.

Liang, P., Lan, F., Lee, A. S., Gong, T., Sanchez-Freire, V., Wang, Y., Diecke, S., Sallam, K., Knowles, J. W., Wang, P. J., *et al.* (2013). Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. Circulation *127*, 1677-1691.
Liem, K. F., Jr., Tremml, G., and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell *91*, 127-138.
Lindvall, O., Brundin, P., Widner, H., Rehncrona, S., Gustavii, B., Frackowiak, R., Leenders, K. L., Sawle, G., Rothwell, J. C., Marsden, C. D., and et al. (1990). Grafts of fetal dopamine neurons survive and improve motor function in Parkinson's disease. Science *247*, 574-577.
Lindvall, O., Rehncrona, S., Brundin, P., Gustavii, B., Astedt, B., Widner, H., Lindholm, T., Bjorklund, A., Leenders, K. L., Rothwell, J. C., *et al.* (1989). Human fetal dopamine neurons grafted into the striatum in two patients with severe Parkinson's disease. A detailed account of methodology and a 6-month follow-up. Arch Neurol *46*, 615-631.
Lindvall, O., Widner, H., Rehncrona, S., Brundin, P., Odin, P., Gustavii, B., Frackowiak, R., Leenders, K. L., Sawle, G., Rothwell, J. C., and et al. (1992). Transplantation of fetal dopamine neurons in Parkinson's disease: one-year clinical and neurophysiological observations in two patients with putaminal implants. Ann Neurol *31*, 155-165.
Liu, A., Losos, K., and Joyner, A. L. (1999). FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development *126*, 4827-4838.
Lo, L., Tiveron, M. C., and Anderson, D. J. (1998). MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. Development *125*, 609-620.
Lowry, W. E. (2012). Does transcription factor induced pluripotency accurately mimic embryo derived pluripotency? Curr Opin Genet Dev *22*, 429-434.
Lyashenko, N., Winter, M., Migliorini, D., Biechele, T., Moon, R. T., and Hartmann, C. (2011). Differential requirement for the dual functions of β-catenin in embryonic stem cell self-renewal and germ layer formation. Nature Cell Biology *13*, 753-761.
Lyness, S. A., Zarow, C., and Chui, H. C. (2003). Neuron loss in key cholinergic and aminergic nuclei in Alzheimer disease: a meta-analysis. Neurobiol Aging *24*, 1-23.
Ma, J., Guo, L., Fiene, S. J., Anson, B. D., Thomson, J. A., Kamp, T. J., Kolaja, K. L., Swanson, B. J., and January, C. T. (2011). High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. Am J Physiol Heart Circ Physiol *301*, H2006-2017.
Maden, M., Gale, E., Kostetskii, I., and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. Curr Biol *6*, 417-426.
Marchetto, M. C., Carromeu, C., Acab, A., Yu, D., Yeo, G. W., Mu, Y., Chen, G., Gage, F. H., and Muotri, A. R. (2010). A model for neural development and treatment of Rett syndrome using human induced pluripotent stem cells. Cell *143*, 527-539.
Marien, M. R., Colpaert, F. C., and Rosenquist, A. C. (2004). Noradrenergic mechanisms in neurodegenerative diseases: a theory. Brain Res Brain Res Rev *45*, 38-78.
Marti, E., Bumcrot, D. A., Takada, R., and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature *375*, 322-325.
Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A *78*, 7634-7638.
Martinat, C., Bacci, J. J., Leete, T., Kim, J., Vanti, W. B., Newman, A. H., Cha, J. H., Gether, U., Wang, H., and Abeliovich, A. (2006). Cooperative transcription activation by Nurr1 and Pitx3 induces embryonic stem cell maturation to the midbrain dopamine neuron phenotype. Proc Natl Acad Sci U S A *103*, 2874-2879.
Medina-Martinez, O., Shah, R., and Jamrich, M. (2009). Pitx3 controls multiple aspects of lens development. Dev Dyn *238*, 2193-2201.
Millonig, J. H., Millen, K. J., and Hatten, M. E. (2000). The mouse Dreher gene Lmx1a controls formation of the roof plate in the vertebrate CNS. Nature *403*, 764-769.

Morin, X., Cremer, H., Hirsch, M. R., Kapur, R. P., Goridis, C., and Brunet, J. F. (1997). Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron *18*, 411-423.
Morrison, J. I., Loof, S., He, P., and Simon, A. (2006). Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population. J Cell Biol *172*, 433-440.
Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. Neuron *23*, 689-702.
Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S. P.*, et al.* (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. Dev Cell *1*, 423-434.
Muroyama, Y., Fujihara, M., Ikeya, M., Kondoh, H., and Takada, S. (2002). Wnt signaling plays an essential role in neuronal specification of the dorsal spinal cord. Genes Dev *16*, 548-553.
Nakagawa, M., Koyanagi, M., Tanabe, K., Takahashi, K., Ichisaka, T., Aoi, T., Okita, K., Mochiduki, Y., Takizawa, N., and Yamanaka, S. (2008). Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol *26*, 101-106.
Nakano, M., Yamada, K., Fain, J., Sener, E. C., Selleck, C. J., Awad, A. H., Zwaan, J., Mullaney, P. B., Bosley, T. M., and Engle, E. C. (2001). Homozygous mutations in ARIX(PHOX2A) result in congenital fibrosis of the extraocular muscles type 2. Nat Genet *29*, 315-320.
Nefzger, C. M., Haynes, J. M., and Pouton, C. W. (2011). Directed expression of Gata2, Mash1, and Foxa2 synergize to induce the serotonergic neuron phenotype during in vitro differentiation of embryonic stem cells. STEM CELLS *29*, 928-939.
Nefzger, C. M., Su, C. T., Fabb, S. A., Hartley, B. J., Beh, S. J., Zeng, W. R., Haynes, J. M., and Pouton, C. W. (2012). Lmx1a allows context-specific isolation of progenitors of GABAergic or dopaminergic neurons during neural differentiation of embryonic stem cells. STEM CELLS *30*, 1349-1361.
Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P., and Dolle, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. Development *127*, 75-85.
Nordstrom, U., Jessell, T. M., and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. Nat Neurosci *5*, 525-532.
Ohi, Y., Qin, H., Hong, C., Blouin, L., Polo, J. M., Guo, T., Qi, Z., Downey, S. L., Manos, P. D., Rossi, D. J.*, et al.* (2011). Incomplete DNA methylation underlies a transcriptional memory of somatic cells in human iPS cells. Nat Cell Biol *13*, 541-549.
Olanow, C. W., Goetz, C. G., Kordower, J. H., Stoessl, A. J., Sossi, V., Brin, M. F., Shannon, K. M., Nauert, G. M., Perl, D. P., Godbold, J., and Freeman, T. B. (2003). A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. Ann Neurol *54*, 403-414.
Oosterveen, T., Kurdija, S., Alekseenko, Z., Uhde, C. W., Bergsland, M., Sandberg, M., Andersson, E., Dias, J. M., Muhr, J., and Ericson, J. (2012). Mechanistic differences in the transcriptional interpretation of local and long-range Shh morphogen signaling. Dev Cell *23*, 1006-1019.
Panchision, D. M., Pickel, J. M., Studer, L., Lee, S. H., Turner, P. A., Hazel, T. G., and McKay, R. D. (2001). Sequential actions of BMP receptors control neural precursor cell production and fate. Genes Dev *15*, 2094-2110.
Panman, L., Andersson, E., Alekseenko, Z., Hedlund, E., Kee, N., Mong, J., Uhde, C. W., Deng, Q., Sandberg, R., Stanton, L. W.*, et al.* (2011). Transcription factor-induced lineage selection of stem-cell-derived neural progenitor cells. Cell Stem Cell *8*, 663-675.
Parish, C. L., Beljajeva, A., Arenas, E., and Simon, A. (2007). Midbrain dopaminergic neurogenesis and behavioural recovery in a salamander lesion-induced regeneration model. Development *134*, 2881-2887.
Patt, S., and Gerhard, L. (1993). A Golgi study of human locus coeruleus in normal brains and in Parkinson's disease. Neuropathol Appl Neurobiol *19*, 519-523.

Pattyn, A. (2000). Specification of the Central Noradrenergic Phenotype by the Homeobox Gene Phox2b. Molecular and Cellular Neuroscience *15*, 235-243.
Pattyn, A., Hirsch, M., Goridis, C., and Brunet, J. F. (2000). Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. Development *127*, 1349-1358.
Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1997). Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development *124*, 4065-4075.
Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature *399*, 366-370.
Pfisterer, U., Kirkeby, A., Torper, O., Wood, J., Nelander, J., Dufour, A., Bjorklund, A., Lindvall, O., Jakobsson, J., and Parmar, M. (2011a). Direct conversion of human fibroblasts to dopaminergic neurons. Proc Natl Acad Sci U S A *108*, 10343-10348.
Pfisterer, U., Wood, J., Nihlberg, K., Hallgren, O., Bjermer, L., Westergren-Thorsson, G., Lindvall, O., and Parmar, M. (2011b). Efficient induction of functional neurons from adult human fibroblasts. Cell Cycle *10*, 3311-3316.
Placzek, M., Yamada, T., Tessier-Lavigne, M., Jessell, T., and Dodd, J. (1991). Control of dorsoventral pattern in vertebrate neural development: induction and polarizing properties of the floor plate. Development *Suppl 2*, 105-122.
Politis, M., Oertel, W. H., Wu, K., Quinn, N. P., Pogarell, O., Brooks, D. J., Bjorklund, A., Lindvall, O., and Piccini, P. (2011). Graft-induced dyskinesias in Parkinson's disease: High striatal serotonin/dopamine transporter ratio. Mov Disord *26*, 1997-2003.
Prakash, N., Brodski, C., Naserke, T., Puelles, E., Gogoi, R., Hall, A., Panhuysen, M., Echevarria, D., Sussel, L., Weisenhorn, D. M.*, et al.* (2006). A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. Development *133*, 89-98.
Ribeiro, D., Laguna Goya, R., Ravindran, G., Vuono, R., Parish, C. L., Foldi, C., Piroth, T., Yang, S., Parmar, M., Nikkhah, G.*, et al.* (2012). Efficient expansion and dopaminergic differentiation of human fetal ventral midbrain neural stem cells by midbrain morphogens. Neurobiol Dis *49C*, 118-127.
Rodaway, A., and Patient, R. (2001). Mesendoderm. an ancient germ layer? Cell *105*, 169-172.
Rodier, P. M., Ingram, J. L., Tisdale, B., Nelson, S., and Romano, J. (1996). Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. J Comp Neurol *370*, 247-261.
Roybon, L., Hjalt, T., Christophersen, N. S., Li, J. Y., and Brundin, P. (2008). Effects on differentiation of embryonic ventral midbrain progenitors by Lmx1a, Msx1, Ngn2, and Pitx3. J Neurosci *28*, 3644-3656.
Sakurada, K., Ohshima-Sakurada, M., Palmer, T. D., and Gage, F. H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development *126*, 4017-4026.
Sanchez-Danes, A., Consiglio, A., Richaud, Y., Rodriguez-Piza, I., Dehay, B., Edel, M., Bove, J., Memo, M., Vila, M., Raya, A., and Izpisua Belmonte, J. C. (2012a). Efficient generation of A9 midbrain dopaminergic neurons by lentiviral delivery of LMX1A in human embryonic stem cells and induced pluripotent stem cells. Hum Gene Ther *23*, 56-69.
Sanchez-Danes, A., Richaud-Patin, Y., Carballo-Carbajal, I., Jimenez-Delgado, S., Caig, C., Mora, S., Di Guglielmo, C., Ezquerra, M., Patel, B., Giralt, A.*, et al.* (2012b). Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO Mol Med *4*, 380-395.
Sandoe, J., and Eggan, K. (2013). Opportunities and challenges of pluripotent stem cell neurodegenerative disease models. Nat Neurosci *16*, 780-789.
Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med *10*, 55-63.
Schwartz, J. W., Blakely, R. D., and DeFelice, L. J. (2003). Binding and transport in norepinephrine transporters. Real-time, spatially resolved analysis in single cells using a fluorescent substrate. J Biol Chem *278*, 9768-9777.

Schwartz, J. W., Novarino, G., Piston, D. W., and DeFelice, L. J. (2005). Substrate binding stoichiometry and kinetics of the norepinephrine transporter. J Biol Chem *280*, 19177-19184.
Sebastiano, V., Maeder, M. L., Angstman, J. F., Haddad, B., Khayter, C., Yeo, D. T., Goodwin, M. J., Hawkins, J. S., Ramirez, C. L., Batista, L. F.*, et al.* (2011). In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. STEM CELLS *29*, 1717-1726.
Si-Tayeb, K., Noto, F. K., Nagaoka, M., Li, J., Battle, M. A., Duris, C., North, P. E., Dalton, S., and Duncan, S. A. (2010). Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. Hepatology *51*, 297-305.
Slack, J. M. (2002). Conrad Hal Waddington: the last Renaissance biologist? Nat Rev Genet *3*, 889-895.
Slawny, N. A., and O'shea, K. S. (2011). Dynamic changes in Wnt signaling are required for neuronal differentiation of mouse embryonic stem cells. Mol Cell Neurosci *48*, 205-216.
Solloway, M. J., and Robertson, E. J. (1999). Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. Development *126*, 1753-1768.
Sonntag, K.-C., Simantov, R., Kim, K.-S., and Isacson, O. (2004). Temporally induced Nurr1 can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation. Eur J Neurosci *19*, 1141-1152.
Spencer, D. D., Robbins, R. J., Naftolin, F., Marek, K. L., Vollmer, T., Leranth, C., Roth, R. H., Price, L. H., Gjedde, A., Bunney, B. S., and et al. (1992). Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease. N Engl J Med *327*, 1541-1548.
Staerk, J., Dawlaty, M. M., Gao, Q., Maetzel, D., Hanna, J., Sommer, C. A., Mostoslavsky, G., and Jaenisch, R. (2010). Reprogramming of human peripheral blood cells to induced pluripotent stem cells. Cell Stem Cell *7*, 20-24.
Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H. (2008). Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. Development *135*, 2969-2979.
Summerhurst, K., Stark, M., Sharpe, J., Davidson, D., and Murphy, P. (2008). 3D representation of Wnt and Frizzled gene expression patterns in the mouse embryo at embryonic day 11.5 (Ts19). Gene Expr Patterns *8*, 331-348.
Swanson, D. J., Zellmer, E., and Lewis, E. J. (1997). The homeodomain protein Arix interacts synergistically with cyclic AMP to regulate expression of neurotransmitter biosynthetic genes. J Biol Chem *272*, 27382-27392.
Tachibana, S., Shimomura, A., and Inadera, H. (2011). Toxicity monitoring with primary cultured hepatocytes underestimates the acetaminophen-induced inflammatory responses of the mouse liver. Tohoku J Exp Med *225*, 263-272.
Tada, S., Era, T., Furusawa, C., Sakurai, H., Nishikawa, S., Kinoshita, M., Nakao, K., Chiba, T., and Nishikawa, S.-I. (2005). Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development *132*, 4363-4374.
Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131*, 861-872.
Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.
Takebe, T., Sekine, K., Enomura, M., Koike, H., Kimura, M., Ogaeri, T., Zhang, R. R., Ueno, Y., Zheng, Y. W., Koike, N.*, et al.* (2013). Vascularized and functional human liver from an iPSC-derived organ bud transplant. Nature.
Taneja, P., Ogier, M., Brooks-Harris, G., Schmid, D. A., Katz, D. M., and Nelson, S. B. (2009). Pathophysiology of locus ceruleus neurons in a mouse model of Rett syndrome. J Neurosci *29*, 12187-12195.
ten Berge, D., Koole, W., Fuerer, C., Fish, M., Eroglu, E., and Nusse, R. (2008). Wnt signaling mediates self-organization and axis formation in embryoid bodies. Cell Stem Cell *3*, 508-518.

ten Berge, D., Kurek, D., Blauwkamp, T., Koole, W., Maas, A., Eroglu, E., Siu, R. K., and Nusse, R. (2011). Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nature Cell Biology *13*, 1070-1075.
Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner, R. L., and McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.
Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.
Tilleman, H., Hakim, V., Novikov, O., Liser, K., Nashelsky, L., Di Salvio, M., Krauthammer, M., Scheffner, O., Maor, I., Mayseless, O*., et al.* (2010). Bmp5/7 in concert with the mid-hindbrain organizer control development of noradrenergic locus coeruleus neurons. Mol Cell Neurosci *45*, 1-11.
Timmer, J. R., Wang, C., and Niswander, L. (2002). BMP signaling patterns the dorsal and intermediate neural tube via regulation of homeobox and helix-loop-helix transcription factors. Development *129*, 2459-2472.
Tiveron, M. C., Hirsch, M. R., and Brunet, J. F. (1996). The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. J Neurosci *16*, 7649-7660.
Tomlinson, B. E., Irving, D., and Blessed, G. (1981). Cell loss in the locus coeruleus in senile dementia of Alzheimer type. J Neurol Sci *49*, 419-428.
Torper, O., Pfisterer, U., Wolf, D. A., Pereira, M., Lau, S., Jakobsson, J., Bjorklund, A., Grealish, S., and Parmar, M. (2013). Generation of induced neurons via direct conversion in vivo. Proc Natl Acad Sci U S A *110*, 7038-7043.
van der Wees, J., Schilthuis, J. G., Koster, C. H., Diesveld-Schipper, H., Folkers, G. E., van der Saag, P. T., Dawson, M. I., Shudo, K., van der Burg, B., and Durston, A. J. (1998). Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain. Development *125*, 545-556.
Vierbuchen, T., Ostermeier, A., Pang, Z. P., Kokubu, Y., Südhof, T. C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035-1041.
Waddington, C. H. (1957). The strategy of the genes; a discussion of some aspects of theoretical biology, (London,: Allen [and] Unwin).
Wagner, J., Akerud, P., Castro, D. S., Holm, P. C., Canals, J. M., Snyder, E. Y., Perlmann, T., and Arenas, E. (1999). Induction of a midbrain dopaminergic phenotype in Nurr1-overexpressing neural stem cells by type 1 astrocytes. Nat Biotechnol *17*, 653-659.
Wallen, A., Zetterstrom, R. H., Solomin, L., Arvidsson, M., Olson, L., and Perlmann, T. (1999). Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. Exp Cell Res *253*, 737-746.
Widner, H., Tetrud, J., Rehncrona, S., Snow, B., Brundin, P., Gustavii, B., Bjorklund, A., Lindvall, O., and Langston, J. W. (1992). Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). N Engl J Med *327*, 1556-1563.
Wray, J., Kalkan, T., Gomez-Lopez, S., Eckardt, D., Cook, A., Kemler, R., and Smith, A. (2011). Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. Nature Cell Biology *13*, 838-845.
Yamada, T., Placzek, M., Tanaka, H., Dodd, J., and Jessell, T. M. (1991). Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. Cell *64*, 635-647.
Yang, C., Kim, H. S., Seo, H., Kim, C. H., Brunet, J. F., and Kim, K. S. (1998). Paired-like homeodomain proteins, Phox2a and Phox2b, are responsible for noradrenergic cell-specific transcription of the dopamine beta-hydroxylase gene. J Neurochem *71*, 1813-1826.
Ying, Q. L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell *115*, 281-292.

Ying, Q. L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P., and Smith, A. (2008). The ground state of embryonic stem cell self-renewal. Nature *453*, 519-523.
Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R.*, et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.
Zarow, C., Lyness, S. A., Mortimer, J. A., and Chui, H. C. (2003). Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol *60*, 337-341.
Zetterstrom, R. H., Solomin, L., Jansson, L., Hoffer, B. J., Olson, L., and Perlmann, T. (1997). Dopamine neuron agenesis in Nurr1-deficient mice. Science *276*, 248-250.
Zhou, Q., Brown, J., Kanarek, A., Rajagopal, J., and Melton, D. A. (2008). In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature *455*, 627-632.
Zou, J., Mali, P., Huang, X., Dowey, S. N., and Cheng, L. (2011). Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood *118*, 4599-4608.
Zweig, R. M., Cardillo, J. E., Cohen, M., Giere, S., and Hedreen, J. C. (1993). The locus ceruleus and dementia in Parkinson's disease. Neurology *43*, 986-991.