Thesis for doctoral degree (Ph.D.) 2010

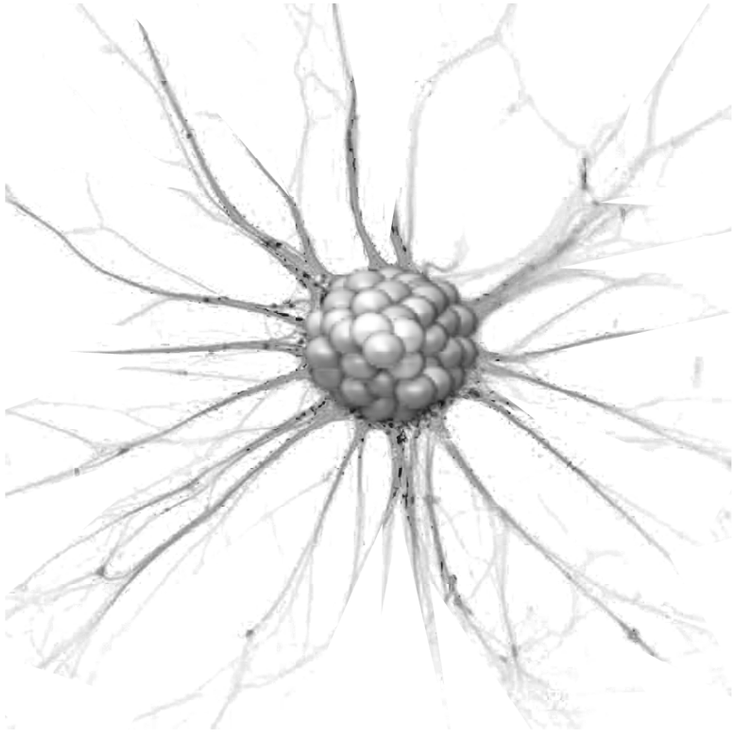
Manipulating Neural Stem Cells

Malin Eriksson

Karolinska Institutet

Thesis for doctoral degree (Ph.D.)
2010

Manipulating Neural Stem Cells



Malin Eriksson

Karolinska Institutet

Department of Cell and Molecular Biology
Karolinska Institutet

MANIPULATING NEURAL STEM CELLS

Malin Eriksson

Karolinska Institutet

Sweden 2010

Published by Karolinska Institutet. Printed by Larserics Digital Prints AB.

ISBN 978-91-7409-853-2

ABSTRACT

The stem cell field is a fairly young but fast growing scientific field. Our understanding of stem cells has changed tremendously over the last couple of decades. This in mainly due to the development of new techniques that has enabled us to study stem cells from a completely new perspective. Several groundbreaking findings were made through technical advances, such as the development of somatic cell nuclear transfer in the 50s and the production of the first cloned mammal, Dolly the sheep in 1996, which demonstrated that nuclei of differentiated cells could be reprogrammed. The development of cultures of adult neural stem cells in the 90s demonstrated the presence of stem cells *in vivo* that could be propagated *in vitro*. More recently, the development of artificial embryonic stem cells, the so called induced pluripotent stem cells (iPS cells), were obtained through the delivery of only four exogenous factors, *Oct4, klf4, Sox2* and *cMyc* to cultured fibroblasts in 2006.

The publications presented in this thesis are based on several of these groundbreaking findings. A common theme in the work of the thesis is the use of neural stem cell for the purpose of addressing biological questions *in vitro* or *in vivo*. **Publication I** describes a new miniaturized platform for culturing embryonic stem cells and adult neural stem cells. This platform contains 672 wells, each one holding 500nL. The volume, large enough to culture stem cells over several days is also small enough to drastically reduce reagent costs. The platform is suitable for large-scale screening, where a large number of substances or culture conditions can be studied simultaneously. **Publication II** presents the development of a rapid and efficient transfection method that allows localized *in vivo* transfection of plasmid DNA within adult neurogenic niches by electroporation. This strategy allows gain-and loss-of function studies as well as fate mapping experiments. Using this approach, we found that cadherins play an essential role for maintaining the integrity of the lateral ventricle wall. Both publication III and manuscript IV aim to further improve the iPS cell production. **Publication III** is one of the first publications where reprogramming of neural stem cells without *Sox2* is reported. The iPS cells generated displayed pluripotency by teratoma formation and production of chimeric mice upon blastocyst injection. Two of the mice developed tumours displaying residual proviral expression. The use of exogenous DNA presents a potential oncogenic risk. Choice of delivery method for the reprogramming factors in combination with reduction of the number of factors required might bring iPS cells closer to therapeutic use. **Manuscript IV** attempts to push adult neural stem cell towards a reprogrammed state through an all-chemical based strategy, omitting the need for exogenous DNA. Adult neural stem cells endogenously express three of the four reprogramming factors. This allows reprogramming of adult neural stem cell through the addition of only one factor, *Oct4*. In manuscript IV *Oct4* expression is chemically induced. Endogenous expression of the pluripotency factors *Nanog* and *Rex1* were measured to investigate whether *Oct4*-inducing conditions could lead to a reprogrammed state.

LIST OF PUBLICATIONS

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

I. Sara Lindström*, **Malin Eriksson***, Tandis Vazin, Julia Sandberg, Joakim Lundeberg, Jonas Frisén and Helene Andersson-Svahn
High-density Microwell Chip for Culture and Analysis of Stem Cells
PLoS One (2009), 4 (9): e6997

II. Fanie Barnabé-Heider, Konstantinos Meletis, **Malin Eriksson**, Olaf Bergmann, Hanna Sabelström, Michael Harvey, Harald Mikkers and Jonas Frisén
Genetic Manipulation of Adult Mouse Neurogenic Niches by in vivo Electroporation
Nature Methods (2008), 5 (2): 189-196

III. Dirk Duinsbergen, **Malin Eriksson**, Peter A.C. 't Hoen, Jonas Frisén and Harald Mikkers
Induced Pluripotency with Endogenous and Inducible Genes
Experimental Cell Research (2008), 314 (17): 3255-3263

IV. **Malin Eriksson**, Justin K. Ichida, Kevin Eggan and Jonas Frisén
Towards Chemical Reprogramming of Adult Neural Stem Cells
Manuscript (2010)

* These authors contributed equally to this work

Related publication not included in this thesis:

Dirk Duinsbergen, Daniela Salvatori, **Malin Eriksson** and Harald Mikkers
Tumors Originating from Induced Pluripotent Stem Cells and Methods for their Prevention
Ann N Y Acad Sci. (2009) 1176:197-204.

TABLE OF CONTENTS

LIST OF SELECTED ABBREVIATIONS

AP	Alkaline phosphatase
AraC	Cytosine-β-D-arabinofuranoside
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
CNS	Central nervous system
DG	Dentate gyrus
EGF	Epidermal growth factor
ES cell	Embryonic stem cell
FGF2	Fibroblast growth factor 2 (or basic-FGF)
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
iPS cell	Induced pluripotent stem cell
Klf4	Krüppel-like factor 4
Oct4	Octamer 4 (or POU5f1)
POU5f1	POU class 5 homeobox 1 (or Oct4)
Rex1	Reduced expression protein 1 (also called zink-finger protein 42, Zfp42)
RG	Radial glia
SCNT	Somatic cell nuclear transfer
SGZ	Subgranular zone
Sox2	Sex determining region Y box 2
SSEA1	Stage-specific embryonic antigen (also called LewisX or LeX)
SVZ	Subventricular zone
RMS	Rostral migratory stream

INTRODUCTION

The work, upon which this thesis is based, concerns *in vitro* and *in vivo* manipulation of mouse neural stem cells and adult neural stem cells.

The term "adult stem cells" is used for stem cells within a specialised tissue. Adult stem cells can be found in almost all tissues of the body. However, they make up a very small fraction of the total cell number. They represent a reserve pool of cells, ready for action when mature cells needs to be replaced due to damage or age.

Adult neural stem cells are stem cells of the central nervous system. Despite having been extensively studied since the beginning of the 90s, specific markers labelling stem cell populations are still lacking. Instead, functional characteristics, localization and a palette of protein markers are often used to describe them. Although these cells might be rare and still undefined, they are interesting from many perspectives, and could answer many biological questions, for example how does the nervous system develop during embryogenesis, which environmental cues do stem cells respond to and what kind of responses are they capable of? Several of these questions return in pathological situations such as, degenerative diseases of the central nervous system, stroke, spinal cord injury and tumour development.

Progress in science is often hampered by technical limitations. Our experiments are restricted by the available methods. The publications, upon which this thesis is based, are related to the development of new techniques or the refining of existing techniques with the aim of manipulating neural stem cells. The assays presented in the publications have different goals with the use of the neural stem cells and adult neural stem cells, the development of a miniaturized platform for large parallel screens *in vitro*, the development of a transfection method allowing labelling and alteration of gene expression in the two germinal regions *in vivo*, to the use of neural stem cells for the purpose of reducing risks in the production of artificial ES cells (iPS cells) and providing information on how pluripotency is maintained.

STEM CELLS

HISTORY, CHARACTERISTICS AND TYPES

The term "stem cells" originates from the German word "Stammzellen" used to describe the concept of stem cells in one of the earliest publications (Askanazy 1907). Stem cells have a unique ability to reproduce themselves as well as give rise to a wide variety of differentiated cell types. In theory, stem cells possess the capability of eternal life. The history of cell biology starts in the middle of the 19th century with the development of microscopy and the discovery that cells were the building blocks of organisms. Stem cell science begins in the middle of the 20th century, when single cells from the bone marrow where found to give rise to different kinds of blood cells, both in rodents and in humans (McCulloch and Till 1960; Thomas, et al. 1957). Initially stem cell based replacement therapies were considered to hold the promise of cure for a wide variety of diseases and malignancies. In particular, blood stem cells were the first to be used for therapeutic purposes, with bone marrow transplants being routinely performed since the 70s as a cure for blood disorders, such as leukaemia, myeloma and immune-deficiency disorders.

Under the name of stem cells we group a wide range of cell types. They are all classified according to their functional characteristics and sub-grouped according to their differentiation potency. The four major groups of stem cells are the totipotent, pluripotent, multipotent and unipotent stem cells. Totipotent cells have the ability to produce all the cell types found in the embryo as well as extra-embryonic tissues, such as the placenta. The only truly totipotent cell is the fertilized egg (zygote). Pluripotent cells are able to give rise to all tissues generating the embryo but no extra-embryonic tissue. To date, there are five types of pluripotent cells known, embryonic stem (ES), embryonic germ (EG), embryonic carcinoma (EC), multipotent germline stem (mGS) and induced pluripotent stem (iPS) cells. All of them can contribute to all cell types of an embryo as well as germline transmission upon tetraploid complementation or injection into a morula or blastocyst. EG and EC cells contribute to a lesser extent than the other cell types. iPS cells are artificially produced pluripotent cells equivalent to ES cells. Multipotent stem cells have the capacity to generate cells within a germ layer (mesoderm, endoderm or ectoderm) or a tissue. Many of the adult stem cells are examples of multipotent stem cells. Unipotent cells can only give rise to cells within

the same lineage. They are considered stem cells, since they exhibit indefinite self-renewing capacity.

EMBRYONIC STEM CELLS

ES cells are derived from the inner cell mass of a blastocyst. The blastocyst is an early embryonic state that occurs 3.5 days post fertilization in mice and 4.5 days post fertilization in humans. The blastocyst is a spherical fluid-filled structure where the inner cell mass consist of a lump of about 20 cells, residing within the blastocyst cavity. These cells will eventually generate the entire embryo. The British scientist Martin Evans was awarded the Nobel Prize in physiology or medicine in 2007 (together with Mario R. Capecchi and Oliver Smithies) for his work on isolation and propagation of mouse ES cells (Evans and Kaufman 1981). The term "embryonic" stem cells was established to distinguish the embryo-derived pluripotent cells from the previously used embryo-derived carcinoma cells (Martin 1981). The derived ES cells were tested for germline transmission and the first evidence of embryo-derived pluripotent cells was published in 1984 (Bradley, et al. 1984).

Almost 30 years have passed since the isolation of the first mouse ES cells. Since then, only few other mammalian species have yielded long-term cultures of embryonic stem cells. The first primate ES cells were derived from a rhesus monkey, by the American scientist James Thomson in 1995 (Thomson, et al. 1995). Afterwards, the same research group derived the first human ES cells (Thomson, et al. 1998). The ultimate potency test for stem cells is whether they have the capacity to give rise to germline transmission upon injection into a blastocyst or morula stage embryo. Germline transmission is regularly tested on rodent ES cells, but cannot be performed on human pluripotent cells for ethical reasons, leaving room for speculations concerning the stem cell capacity of human pluripotent stem cells. Stem cells are believed to hold great promise for therapeutics. Therefore major efforts are being made in order to improve culture conditions and establish ES cell lines suitable for transplantation.

ADULT STEM CELL

The term “adult stem cells” is used for stem cells within a specialised tissue. Adult stem cells are multipotent and found in almost all kinds of tissues. Their function is tissue repair and maintenance of homeostasis within the organ they belong to. Adult stem cells are rare often located in special niches that create a suitable environment for the stem cell populations. The niche has the major task of regulating the stem cell pool, maintaining the population and at the same time responding to the needs of the surrounding tissue.

The first adult stem cells were discovered in the late 50s in the bone marrow (Thomas, et al. 1957). Today, we know that adult stem cells reside within almost all organs in the human body. Adult stem cells are multipotent cells with diverse proliferative capability. Organs subjected to harsh environments, such as skin, gut and blood have stem cells with much higher proliferative capacity than for example muscle stem cells and neural stem cells found in the CNS.

Using adult stem cells for scientific or therapeutic purposes is less controversial in comparison to ES cells. Nowadays, adult stem cells are the only stem cells commonly used in therapeutics.

ADULT NEURAL STEM CELLS

THE DISCOVERY OF MAMMALIAN NEUROGENESIS

It is a well-accepted concept that non-mammalian vertebrates, like fish, newts and birds have ongoing neurogenesis throughout adulthood (Brockes 1997; Johns and Easter 1977; Nottebohm, et al. 1990). Vertebrate classes such as amphibians and fish have amazing regenerative capacity, this has been illustrated by the severing of legs, optical nerves and even spinal cord (Becker, et al. 1997; Brockes 1997; Johns and Easter 1977). However, mammals in general, and humans in particular, were believed to have lost this capacity as evolution progressed, this was especially true when it comes to the CNS. The primate and the human brain were considered to be too sophisticated and complex to have maintained such a primitive but useful function. Ramon y Cajal, one of the most famous neuroscientist and Nobel prize laureate in 1906, stated that "*Everything may die, nothing may be regenerated*" (Cajal 1913). This dogma has changed slowly over the years. In fact, the first publication reporting the presence of neurogenesis in the adult mammalian brain by Altman and Das was not well received (Altman and Das 1965). By using a thymidine analogue (tritiated thymidine) that incorporates in the genome of dividing cells, they demonstrated that there is an addition of neurons in the hippocampal area of adult rats. Altman confirmed, using the same labelling method, that new cells are born after birth also in the olfactory bulb (OB) of adult rats (Altman 1969). However, limitations of the methods at that time made it impossible to state for sure that the labelled cells were indeed neurons and the publications were therefore discarded by the majority of neuroscientists at that time. It took until the beginning of the 80s before the morphology of the tritiated thymidine labelled cells could be studied in more detail by electron microscopy (Kaplan 1981). The year after, the development of a new thymidine analogue, 5-bromodeoxyuridine (BrdU) (Gratzner 1982), made it possible to co-label the proliferative cells with cell-type specific markers. Sceptics still argued that the BrdU labelled cells were not neurons and that BrdU labelling could be a sign of DNA-repair rather than cell turnover. One long lasting argument for the absence of adult neurogenesis was that a stable non-proliferative neuronal circuit was necessary for the maintenance of learning and long-term memory formation (Rakic 1985a; Rakic 1985b).

In vitro studies performed in the early 90s demonstrated that cells from forebrain regions could be propagated over time as free-floating clusters, so-called neurospheres. These neurospheres could give rise to secondary spheres clonally, displaying self-renewing behaviour. They also displayed multipotent features, generating the three main subtypes of cells found in the nervous system, neurons, astrocytes and oligodendrocytes upon differentiation (Reynolds, et al. 1992; Reynolds and Weiss 1996; Richards, et al. 1992). These experiments showed that cells with neurogenic stem cell or progenitor capacity (at least *in vitro*) resides in the adult mammalian brain.

Adult neurogenesis has been mostly studied in rodents over the years, but studies also in non-human primates and humans have demonstrated the presence of adult neurogenesis (Curtis, et al. 2007; Doetsch and Alvarez-Buylla 1996; Eriksson, et al. 1998; Kornack and Rakic 1999; Lois and Alvarez-Buylla 1993; Lois and Alvarez-Buylla 1994; Sanai, et al. 2004). Nowadays, it is widely accepted that proliferative regions exist in the adult mammalian brain and that there are two major sites of neurogenesis, the subventricular zone/olfactory bulb (SVZ/OB) region and the dentate gyrus (DG) of the hippocampus. Adult neurogenesis replaces and adds neurons to the OB and hippocampus. This maintains the function of the tissue as well as providing plasticity to the organ (Garthe, et al. 2009; Imayoshi, et al. 2008; Magavi, et al. 2005; Winner, et al. 2002). The SVZ/OB and the DG are not solely neurogenic niches. Both regions are able to produce cells of the glial lineage. SVZ/OB give rise to oligodendrocytes of the corpus callosum, fimbria and striatum (Jackson and Alvarez-Buylla 2008) and the DG gives rise to subgranular zone astrocytes (Suh, et al. 2007).

ADULT NEURAL STEM CELLS ORIGIN AND IDENTITY

Neurogenesis occurs between E12 and E20 in the rodent cerebral cortex. Glial cells, astrocytes and oligodendrocytes, are formed at later stages, around E16 and onwards. The forebrain is generated from neuroepithelial cells residing in the basal neuroepithelium along the anterior-posterior neuroaxis. Neuroepithelial cells are elongated cells that make contacts with both the ventricle and the pial surface of the developing brain. As cortex thickens during the progression of embryogenesis, the neuroepithelial cells are stretched out maintaining their bipolar contacts. These extended cells most likely transform into radial glia cells, the major stem cell

population of the embryonic and postnatal brain (Anthony, et al. 2004; Merkle and Alvarez-Buylla 2006; Seri, et al. 2004). Radial glial cells give rise to interneuron-producing cells in the adult mouse brain, establishing a link between these embryonic precursor cells and the neural stem cells of the adult germinal zones (Merkle and Alvarez-Buylla 2006; Merkle, et al. 2004; Seri, et al. 2004). The radial glial cells have also been demonstrated to be the source of ependymal cells in the adult lateral wall (Spassky, et al. 2005).

Establishment of neural stem cell identity is of considerable interest for the understanding of adult neural stem cells and their role in health and disease. This is hampered by the lack of an unambiguous stem cell marker, making it difficult to solely study the stem cells populations. Today, neural stem cells are defined by a set of markers and functional characteristics, such as localization, morphology, cell cycle properties and BrdU incorporation together with the stem cell functional characteristics; multipotency and self-renewal. Marker proteins associated with the adult neural stem cells are Nestin (Lendahl, et al. 1990), Sox2 (D'Amour and Gage 2003), BLBP (Rousselot, et al. 1997), Prominin1 (Coskun, et al. 2008; Uchida, et al. 2000) and GFAP (Doetsch, et al. 1999a; Doetsch, et al. 1999b). However, none of these markers are selective enough to specifically pinpoint the multipotent neural stem cell population. The neural stem cell population in the SVZ shares several features with radial glia-like astrocytes and ependymal cells. They express the mature astrocyte marker GFAP and the ependymal and stem cell marker Prominin1 (Marzesco, et al. 2005; Weigmann, et al. 1997) and in many cases are found intermingled between the ependymal cells in the lateral wall extending one single cilia into the ventricle (Doetsch, et al. 1999a; Luo, et al. 2008; Mirzadeh, et al. 2008; Shen, et al. 2008). The role of the mature astrocyte marker GFAP as a stem cell marker has been shown through labelling GFAP-expressing subpopulations, either virally or through transgenic approaches (Doetsch, et al. 1999a; Garcia, et al. 2004; Imura, et al. 2003). These studies conclude that GFAP-positive cells are proliferative and display a bipolar morphology different from mature GFAP-positive astrocytes and that these cells can give rise to mature neurons populating both the OB and the hippocampus (Doetsch, et al. 1999a; Garcia, et al. 2004; Laywell, et al. 2000; Seri, et al. 2001).

NEUROGENESIS IN THE SVZ/OB SYSTEM

The subventricular zone (SVZ) is the largest germinal zone in the adult CNS. It comprises a thin area, located along the entire lateral ventricle wall covering the striatum. The SVZ harbours the neural stem cells and neuronal precursors generating newborn neurons destined for the OB. The SVZ/OB system is a highly productive region in rodents, producing between 30 000-60 000 new cells per day (Biebl, et al. 2000; Lois and Alvarez-Buylla 1994). The majority of these neurons will not survive and be functionally integrated into a neuronal circuit (Winner, et al. 2002). This excessive production resembles very much the neurogenic behaviour during embryogenesis (Oppenheim 1991).

The neural stem cell niche is closely associated with an extensive blood vessel system found along the lateral wall of the lateral ventricle (Shen, et al. 2008; Tavazoie, et al. 2008). This enables close contact between the cells in the niche and the immediate surrounding. The SVZ niche comprises at least four different kinds of cells, ependymal cells, neural stem cells, transient amplifying cells and neuroblasts. The *ependymal cells* are multiciliated cells lining the lateral wall that separates the subventricular region from the cerebrospinal fluid in the lateral ventricle. They provide a barrier as well direct signal molecules and promote neuroblasts migration along the lateral wall with their beating cilia (Mirzadeh, et al. 2010; Sawamoto, et al. 2006). The ependymal cells are post-mitotic under physiological conditions (Bruni 1998; Bruni, et al. 1985; Carlen, et al. 2009; Doetsch, et al. 1999b; Spassky, et al. 2005). The *neural stem cells* (also called type B cells) reside juxtaposed to the ependymal cell layer. They are often described as astroglial-like, expressing GFAP and a palette of other markers, none of them specific for the adult neural stem cell population. Type B cells, represent a relatively quiescent population that give rise to a highly proliferative, *transient amplifying population* (type C cells) (Doetsch, et al. 1999a). The transient amplifying population is the main population labelled with proliferative markers such as BrdU, Ki67 and proliferating cell nuclear antigen (PCNA). This population expresses protein markers such as Dlx2, Mash1 and FGFR. These intermediate progenitors give rise to PSA-NCAM-positive immature *neuroblasts* (type A cells) that migrate tangentially in chains through the rostral migratory stream (RMS) to the OB. The RMS is a tube-like structure formed by astrocytes separating the migrating neuroblasts from the surrounding parenchyma. Once in the OB, the neuroblasts start migrating radially into different layers of the OB, where they differentiate into the two main types of

GABAergic interneurons, the granule interneurons and the periglomerular interneurons (Goldman 1995; Lois and Alvarez-Buylla 1993; Luskin 1993).

The type of interneurons formed is decided already at their origin in the SVZ, long before the immature neuroblasts have reached the OB. Different regions of the SVZ have been found to generate different kinds of interneurons. This regional specificity is maintained even when cells are heterotopically grafted. Anterior and dorsal regions of the SVZ mainly give rise to periglomerular interneurons and dorsal regions to superficial granule interneurons, whereas the ventral regions mainly give rise to deeper granule interneurons (Merkle, et al. 2007). A recent study has also demonstrated that glutamatergic interneurons can be formed from neural stem cells in the dorsal regions of the SVZ (Brill, et al. 2009).

NEUROGENESIS IN THE HIPPOCAMPUS

New neurons are formed in the hippocampus throughout the lifetime of the organism, although somewhat declining with age (Kuhn, et al. 1996). About 9 000 new cells per day have been reported in young adult rats (Cameron and McKay 2001) and about 10-times less in adult primates (Kornack and Rakic 1999). The neural stem cells of the hippocampus reside in the so-called subgranular zone (SGZ) of the DG, at the border of the hilus and the granule cell layer of the DG (Seri, et al. 2004). The stem cells of the hippocampus display astrocytic features much like the SVZ neural precursor cells. They express GFAP and have long radial processes making close contacts with blood vessels (Palmer, et al. 2000). The neural precursor population, also called type-1 cells or B-cells, have been further subdivided into two types the radial astrocytes (rA) and the horizontal astrocytes (hA) (Seri, et al. 2004; Suh, et al. 2007). The rAs have a round or pyramidal cell body and a radial process that penetrates the granule cell layer and extending towards surface of the DG. This long radial process is branched in the molecular layer. On the other hand, the hAs exhibit no radial process, but a thin basal lamellae oriented tangentially along the SGZ. They commonly express astrocytic and radial glial markers such as Sox2, Vimentin, Blbp and Musashi1. Fate-mapping studies on Sox2-expressing populations have demonstrated that they give rise to neuroblasts that migrate to the granule cell layer, where they integrate and form glutamatergic excitatory dentate granule cell neurons (Suh, et al. 2007). Furthermore, this study showed that the Sox2-positive population also gave rise to SGZ astrocytes and identical

Sox2-positive cells, consistent with true stem cells features such as, multipotency and self-renewal *in vivo*.

The maturing neurons migrate a short distance to the granule cell layer where they predominantly generate local glutamatergic excitatory granule neurons. The maturing granule neurons extend an axon, called mossy fiber, into the CA3 region of the hippocampus (Hastings and Gould 1999) and dendrites into the molecular layer of the DG. The mossy fibers contact inhibitory interneurons of the granule cell layer, hilus and CA3 area as well as excitatory mossy cells in the hilus and CA3 pyramidal cells (Henze, et al. 2000). Neurons have been documented to be fully integrated and functional about 4 weeks post cell division (van Praag, et al. 2002; Zhao, et al. 2006). At this stage, they are morphologically undistinguishable from pre-existing granule neurons (Kempermann, et al. 2003).

The hippocampus is a well-studied region with an undisputable role in learning and memory formation. The anatomical regions of the hippocampus seem to play somewhat different roles. The dorsal part of the hippocampus is involved in learning and memory formation and the ventral part of the hippocampus have been shown to take part in affective behaviour (Bannerman, et al. 2004). Hence, regulation of neurogenesis in the hippocampus has been connected to learning, memory formation and regulation of mood (Garthe, et al. 2009; Kee, et al. 2007; Warner-Schmidt and Duman 2006; Zhao, et al. 2008).

CULTURING OF ADULT NEURAL STEM CELLS

The first cultures of adult neural stem cells were performed in the early 90s (Reynolds, et al. 1992; Reynolds and Weiss 1992). These pioneering publications proved that precursor cells could be found *in vivo* and propagated *in vitro* as free-floating clusters, neurospheres. The hallmarks of stem cells, self-renewal and multipotency are based on these primary findings. Neural stem cells self-renew clonally while kept in FGF2 and EGF. The removal of mitogens results in the displaying of their multipotent properties, giving rise to the three main cell types of the CNS, neurons, astrocytes and oligodendrocytes. Neurosphere assays are still very much in use today, with minor modifications to the original protocols. In addition to neurospheres, neural stem cells can also be cultured as adherent monolayers on matrix (Pollard, et al. 2006).

Neural stem cell cultures are derived from a single-cell suspension isolated from the region of interest. The cell suspension is maintained in uncoated tissue culture dishes under serum-free conditions with supplement of mitogens. Neural stem cell cultures have been successfully derived from many regions along the neuroaxis of the CNS, from the OB to the spinal cord (Golmohammadi, et al. 2008; Gritti, et al. 2002; Weiss, et al. 1996). They can be isolated from human, mouse and rat, embryonic and adult CNS.

Adult neural stem cells from the mouse SVZ/OB region typically express the protein marker Sox2, Nestin, Blbp, RC2, SSEA1, musashi1 and EGFR. However, cells displaying such markers are intermingled with cells expressing markers of more mature cell types, such as PSA-NCAM and beta-tubulin III (Kaneko, et al. 2000; Suslov, et al. 2002). A neurospheres is a compact cluster of cells, sometimes displaying cilia-like cytoplasmatic protrusions from the surface. The inner core is composed of more differentiated cells and depending on the sphere size, even necrotic cells. This might be due to a lower access to the oxygen and mitogens present in the medium (Bez, et al. 2003; Ostenfeld, et al. 2002). This is a limitation of the sphere cultures in comparison to adherent neural cultures, where all cells have the same access to nutrition and metabolite exchange. However, compact spheres formed under free-floating conditions are also believed to create a niche for stem cells. Therefore they may resemble the *in vivo* situation closer than the adherent cultures.

Neural stem cell cultures have provided valuable information about neurogenesis within the mammalian nervous system. However, *in vitro* culturing systems have also limitations. Cultures are sensitive to the culturing methods, such as the frequency and method of passaging, concentration of factors in the medium and overall number of passages (Caldwell, et al. 2001; Irvin, et al. 2003; Morshead, et al. 2002). The composition of the sphere cultures also changes with the overall size of the sphere, containing a mixture of stem cells, progenitor cells and cells at different stages of maturation. The heterogeneity increases with sphere size (Parmar, et al. 2003; Reynolds and Weiss 1996; Suslov, et al. 2002). Cell cultures are highly artificial; the neural stem cells are extracted and propagated without the instructive cues from their niche. Culture conditions are also fairly generous, allowing all dividing cells to be propagated (Seaberg and van der Kooy 2003). This results in primary spheres composed of a mixture of cells, where many of the cells have the ability to clonally form secondary spheres. The majority of the secondary spheres will be derived from progenitor cells and not from a stem cell. All these factors have led to some

discrepancies between the data of different research groups, sometimes making comparison difficult.

Extended culturing of any stem cell population is not advisable. This might lead to overall changes in the composition of the culture. This is also true for adult neural stem cells, where increased proliferative capacity at the expense of differentiation capacity has been reported. The neural stem cell cultures display a bias towards astrocytic fate with reduced capacity to generate neurons and oligodendrocytes after long term culturing (Chang, et al. 2004; Vukicevic, et al. 2010). This might be caused by selection from the culture conditions, by fast dividing cells over multipotent cells or by a general loss of stem cell capacity over the number of passages. Extended culturing also increases the risk for chromosome instability (Ferron, et al. 2004; Vukicevic, et al. 2010) and overall alterations in the gene expression. The later is still controversial. Some publications report that the intrinsic regional specification is maintained or partially maintained (Hitoshi, et al. 2002; Ostenfeld, et al. 2002; Parmar, et al. 2002) whereas others report the opposite (Hack, et al. 2004; Santa-Olalla, et al. 2003). This might be due to differences in culture conditions as well as regional differences within the CNS.

Despite these limitations, neural stem cell cultures have several advantages, making them useful as tools in stem cell biology. Neural stem cell cultures, adherent or free-floating, provide an excellent platform for expansion of adult neural stem cells. The cultured cells are easily manipulated through plasmid transfection or virus infection allowing for loss-of- function or gain-of-function experiments as well as fate-mapping experiments. The neural stem cell cultures can be expanded and investigated further, for stem cell characterisation, as model system for differentiation and intrinsic specification between stem cell populations, both regional and temporal. The culture conditions are defined serum-free conditions, allowing studies on how different cells respond to any extrinsic cue added to the culture medium.

Neural stem cell cultures play a pivotal role in the publications presented in this thesis. Publications I and IV are based on neurosphere cultures, adherent adult neural cultures or both. The work in publication III was also based on neural stem cells. However, these were differentiated from ES cells through exposure to neural stem cell medium (Conti, et al. 2005).

INDUCED PLURIPOTENT STEM CELLS

NUCLEAR REPROGRAMMING

An organism consists of many different and specialised cell types. However, all of them are originally derived from one single cell, the fertilized egg (zygote). The zygote is the ultimate stem cell, with the capability to generate the entire organism as well as extra-embryonic tissue, such as the placenta. The fact that one single cell can contain all the information necessary for all cell types is not so surprising, but the fact that all cells of the adult body maintain identical genetic information (with exception for mature antibody producing cells) is perhaps more astonishing. This was first demonstrated by some elegant experiments by the British scientist John Gurdon in the 60s. He showed that frog eggs with nuclei from the skin or the intestinal epithelium could give rise to live tadpoles (Gurdon 1962; Gurdon and Uehlinger 1966). His experiments concluded that the differentiation process does not cause any irreversible changes to the genome, despite the great variation in morphology and functionality among mature cells. Gurdon's experiments were preceded by the development of a method called somatic cell nuclear transfer (SCNT), where a naked nucleus was transferred into an enucleated egg (Spemann 1938). This method reached a technical breakthrough when Robert Biggs and Thomas King demonstrated that the nuclei from early frog blastocyst stage embryos could generate live tadpoles upon injection into enucleated eggs, leading to the world's first cloned animal, a tadpole (Briggs and King 1952).

It would then take until 1996 before the world's first mammal was cloned, Dolly the sheep (Campbell, et al. 1996). Dolly was cloned using SCNT, from a cultured mammary gland cell, taken from an adult sheep. The cloning of Dolly caused a world sensation and was over the years, followed by numerous publications reporting a range of cloned animals, such as cow, mouse, pig, cat, horse and dog (ABS global, 1997; (Galli, et al. 2003; Lee, et al. 2005; Polejaeva, et al. 2000; Shin, et al. 2002; Wakayama, et al. 1998). This proved that the differentiation process could be reversed even for more complex animals than frogs.

The closest scenario to cloning humans (which for obvious reasons is not allowed) would be the successful derivation of primate ES cells from an adult monkey using SCNT. Indeed this was successfully performed in 2007 (Byrne, et al. 2007).

Animal cloning can answer many questions concerning how pluripotency is restored and maintained. However, animal cloning has a very low success rate and requires the questionable use of oocytes. On the other hand, cell fusion experiments might represent a more straightforward method to study the plasticity of a differentiated stage (Blau and Blakely 1999). Cell fusion is, as the name implies, two cells fused at the level of cytoplasm, with the nuclei either remaining separate or fused. This has shown that the more dominant and often larger nucleus overwrites the transcriptional profile of the more submissive nucleus and imposes its own gene expression pattern onto the other one. This is the case for both the fusion of two somatic cells (Harris 1967; Pomerantz and Blau 2004) and for the fusion of a somatic cell with a pluripotent cell. The phenotype of the less differentiated nucleus reverts the transcript of the mature cell nucleus to a stem cell-like expression pattern (Cowan, et al. 2005; Miller and Ruddle 1976; Tada, et al. 2003; Tada, et al. 2001; Yu, et al. 2006). The still open question was, whether it was the nucleus or the cytoplasm of the pluripotent cell that mediated this effect? The problem was solved by an elegant experiment, where the nuclei and the cytoplasm of ES cells were separated and fused with cells isolated from neurosphere cultures. The neurosphere cells fused only with ES cell cytoplasm did not display any reprogramming. However, neurosphere cells fused with the ES cell nucleus only did exhibit nuclear reprogramming, concluding that the reprogramming effect is a nuclear mediated effect (Do and Scholer 2004). The reprogramming of human somatic cells is an attractive strategy to acquire patient specific cell lines, which can be customized for cell replacement therapies. However, fused cells, although pluripotent, are tetraploid, less viable and have lower proliferative capacity than normal cells. The method is also quite inefficient and will therefore not be the sought as a solution in cell replacement therapy. Despite this, cell fusion experiments have demonstrated that cells can maintain and constantly instruct themselves and daughter cells to stay in a lineage and that nuclear reprogramming can occur without cell division (Bhutani, et al. 2009; Do and Scholer 2004).

Both SCNT and cell fusion experiments prove that the epigenetic status of a cell can be reset to a pluripotent state. However, both these experimental set ups requires the use of either oocytes or ES cells, both controversial cell sources.

NUCLEAR REPROGRAMMING BY DEFINED TRANSCRIPTION FACTORS

In September 2006 Takahashi and Yamanaka presented a groundbreaking publication where they described the production of pluripotent, ES cell-like, cells derived from mouse tail-tip fibroblasts (Takahashi and Yamanaka 2006). They proved that ectopic overexpression of only four key transcription factors, *Oct4*, *Sox2*, *Klf4* and *cMyc*, in combination with *Fbx15* selection (an *Oct4* target gene), was sufficient to induce reprogramming of these terminally differentiated adult cells. The new stem cells were named induced pluripotent stem cells, or iPS cells, and they displayed pluripotency through their ability to generate teratomas, but they were unable to generate live chimeras. This problem was solved the year after, using more stringent selection criterias for the selection of reprogrammed cells (Nanog or Oct4), resulting in iPS cells undistinguishable from ES cells and in successful live chimera production (Maherali, et al. 2007; Okita, et al. 2007; Wernig, et al. 2007). This remarkable reprogramming was not exclusive for fibroblasts, but also for example B-cells, stomach cells, liver cells and pancreatic β-cells were reprogrammed using the same quartet of transcription factors (Aoi, et al. 2008; Hanna, et al. 2008; Stadtfeld, et al. 2008a).

Only one year after Yamanaka´s sensational publication, two independent research groups reported the reprogramming of human somatic cells, also fibroblasts with either the four factors published by Yamanaka or by a combination of Oct4, Sox2, Nanog and LIN28 (Takahashi, et al. 2007; Yu, et al. 2007). These iPS cells were tested for pluripotency by teratoma formation assays.

The discovery of direct reprogramming of somatic human cells introduced a whole new world of possibilities. The method was free from the questionable use of oocytes and ES cells and successful in generating pluripotent cells from a wide variety of somatic cell types. Direct reprogramming moved science one step closer to customized cells for replacement therapy, without the risk of rejection as well as provided a source for patient-specific ES-like cell lines for disease modelling and drug screening. However, there were some major safety issues involved in the creation of iPS cells according to the original Yamanaka-version of reprogramming. First was the tumorigenic concern, the use of oncogenic factors and the retroviral choice of delivery and second, the low efficiency in the production of iPS cells. The oncogenic factors used in the original Yamanaka reprogramming cocktail were *cMyc* and *Klf4*. These are two known oncogenes and studies on mice produced by iPS cells or

their offspring have demonstrated fast growing tumours upon their reactivation (Duinsbergen, et al. 2008; Huangfu, et al. 2008b; Nakagawa, et al. 2008; Okita, et al. 2007). *cMyc* was early found to be dispensable among the four factors in the reprogramming process (Aoi, et al. 2008; Nakagawa, et al. 2008; Wernig, et al. 2008b; Yu, et al. 2007). *Klf4* has more recently been successfully omitted in the reprogramming process, requiring either VPA or the addition of a third reprogramming factor *Esrrb* (Feng, et al. 2009; Lyssiotis, et al. 2009). Reprogramming without *cMyc* and *Klf4* is less efficient, why these genes are considered to play a facilitating role in the reprogramming process (Nakagawa, et al. 2008; Wernig, et al. 2008b). Another tumorigenic concern was raised by the choice of delivery method used to introduce the exogenous transcription factors. Retroviruses have the advantage of stable integration and high expression of the reprogramming factor but there is always a risk of causing insertional mutagenesis. Therefore, non-integrating strategies to deliver the reprogramming factor were soon investigated. Strategies using non-integrating viruses, such as adenoviruses (Stadtfeld, et al. 2008b; Zhou and Freed 2009), and complete virus-free strategies using plasmid-based delivery systems followed (Kaji, et al. 2009; Okita, et al. 2008). However, all the methods mentioned so far require the addition of exogenous DNA. To my knowledge, there is only one publication that presents a non-exogenous DNA reprogramming strategy, where they use recombinant cell-penetrating proteins of the four factors, Oct4, Klf4, Sox2 and cMyc to mediate the reprogramming (Zhou, et al. 2009).

The first four factor-induced reprogramming protocols had a very low success rate, with only 1 out of ~2 000 somatic cells successfully reprogrammed (Okita, et al. 2007). This had to be addressed before iPS cells could really become a reliable source of stem cells. Today there is a long list of chemicals, culture conditions and gene expression modifications that not only accelerate the reprogramming process but also can substitute for one or two of the original reprogramming factors. Chemicals shown to enhance the efficiency are for example VPA (Huangfu, et al. 2008b), AZA (Mikkelsen, et al. 2008), BIX-01294 (Kubicek, et al. 2007; Shi, et al. 2008a; Shi, et al. 2008b), vitamin C (Esteban, et al. 2009) or a combination of MEK-inhibitor (PD0325901) and GSK-3-inhibitor (CHIR99021) (Silva, et al. 2008). Hypoxic culture condition was also reported to improve the reprogramming efficiency (Yoshida, et al. 2009). In addition to the reprogramming factor, manipulation of gene expression has also greatly enhanced the reprogramming efficiency. Genes promoting reprogramming by overexpression are, for example, Nanog and Lin28 (Hanna, et al. 2009), and genes

that promote reprogramming by downregulation of their expression are for example, p53, p21 (Hanna, et al. 2009; Hong, et al. 2009) and Ink/Arf (Li, et al. 2009). Gene expression can also be modified by microRNAs. MicroRNAs are short non-coding single-stranded RNAs. They are a part of the gene regulatory machinery, by binding to messenger RNAs and committing them to degradation, deadenylation or transcriptional repression (Bagga, et al. 2005; Du and Zamore 2005; Zhao and Srivastava 2007). MicroRNAs are involved in tissue specification and maintenance, most likely through the repression of key genes for undesired differentiation fates. This has been described in particular for ES cells maintenance (Chen, et al. 2007; Ivey, et al. 2008). The role of microRNAs in reprogramming is not completely understood. However, there are a few microRNAs that have been implicated in the reprogramming process. Overexpression of miR-320 in human cancer cells, for example, have led to reprogrammed human cells (Lin, et al. 2008). *Lin28*, one of the reprogramming factors, might regulate pluripotency directly through the regulation of the *Let7* family of microRNAs (Newman, et al. 2008; Viswanathan, et al. 2008) and the overexpression of mouse miR-291, miR-294 and miR-295 can increase the reprogramming efficiency of *Oct4, Sox2* and *Klf4* transduced mouse fibroblast but not in combination with *cMyc*. The result might suggest that one of the roles for *cMyc* is regulating transcriptional activation of these mouse ES cell specific microRNAs (Judson, et al. 2009).

Major events in the reprogramming process

How the reprogramming process occurs is still largely unknown. The exact time scale and sometimes order of critical events is hard to state since it is a gradual and not a step-wise process and also because of the heterogeneity of the cells undergoing reprogramming. Despite being a gradual process, some events must occur to establish pluripotency. (1) Induction of cellular proliferation, most likely mediated through the exogenous transcription factor *cMyc.* It is believed to play a role in the initial process of reprogramming (Sridharan, et al. 2009). *cMyc* has been found to accelerate the cell-cycle progression through G1 by positively regulating the cyclin-Cdk complex (Amati, et al. 1998). More recent studies of *cMyc* and its role in reprogramming have shown that *cMyc* directly regulates the expression of microRNAs involved in regulating cell-cycle progression through regulation of key cell-cycle molecules such as cyclin D2 and p21 (Judson, et al. 2009; Lee, et al. 2008; Wang, et al. 2008). (2) Resetting of the chromatin structure, DNA methylation and histone modifications in part mediated by

proliferation. This gradual relaxation of the chromatin structure enables the exogenous pluripotency factors *Sox2, Oct4* and *Klf4* to access necessary target genes in order to establish an "in house" pluripotency network. *Oct4* and *Sox2* dimerize and co-occupy, often together with *Klf4*, the promoter region of several pluripotency factors including their own promoters (Boyer, et al. 2005; Kim, et al. 2008b; Masui, et al. 2007). Histone methylation plays a critical role in the reestablishment of pluripotency. Global histone methylation patterns need to be restored to an ES cell-like state. This is at least in part mediated through *Oct4, Sox2* and *Nanog* co-occupying promoter and recruiting polycomb group proteins, leading gene expression alteration through histone methylations (Lee, et al. 2006). The *Oct4* and *Sox2* complex have also been found to directly regulate the transcription of several chromatin-remodeling factors such as histone demetylaeses (Loh, et al. 2006; Loh, et al. 2007). (3) A more stem cell-like overall chromatin structure results in a step-wise downregulation of tissue specific genes (such as *thy1* in fibroblasts) in parallel with (4) step-wise upregulation of embryonic markers such as alkaline phosphatase and stage-specific embryonic antigen-1 (*SSEA-1*) and later on induction of endogenous pluripotency genes, *Oct4, Nanog* and *Sox2* (Okita, et al. 2007; Wernig, et al. 2008b; Wernig, et al. 2007). (5) Silencing of the exogenous factors by upregulation of *de novo* DNA methyltransferases Dnmt3a and Dnmt3b, once again directly regulated by *Oct4, Sox2* and *Nanog* along with other transcription factors (Sinkkonen, et al. 2008). Meanwhile, maintaining the endogenous factors by a self-sustainable pluripotency network. (6) Reactivation of the inactive X chromosome (in female cells) through repression of *Xist*. This also mediated directly through the binding of the core pluripotency transcription factors, *Oct4, Nanog* and *Sox2,* to the *Xist* locus (Do, et al. 2008; Maherali, et al. 2007). The generated iPS cells should be capable of giving rise to new iPS daughter cells over a sustained period as well as displaying true pluripotency in order to be considered *bona fide* stem cells.

Reprogramming for everyone?

Various cell types, from mouse, rat, monkey and humans, have been successfully reprogrammed. Nevertheless, the efficiency and the number of factors required vary with cell type and only a small portion of the cells exposed to reprogramming factors becomes pluripotent. A larger portion of the cells seems to become stuck in a partially reprogrammed state dependent on a continuous expression of the extrinsic

reprogramming factors (Mikkelsen, et al. 2008; Silva, et al. 2008; Takahashi and Yamanaka 2006). Is the low reprogramming efficiency due to some intrinsic differences that make some cells more prone to dedifferentiate or is it only a cell culture artifact? Can all cell types be reprogrammed or can only a subset of cells in a population or organ dedifferentiate to a fully pluripotent state, an elite population? Previous SCNT studies demonstrate that less differentiated cells, such as tissue specific stem cells, reprogram easier than fully mature cells (Blelloch, et al. 2006). Similar results were obtained recently in a reprogramming assay using hematopoietic cells at different stages of maturation (Eminli, et al. 2009). The immature hematopoietic stem and progenitor cells were up to 300 times easier to reprogram than terminally differentiated B and T cell. This does not support the conclusion that only a subset of cells can be reprogrammed or that a more mature differentiation state might be a barrier for reprogramming, rather it would be more consistent with an elite model.

The fact that fully differentiated cells have been reported to reprogram would speak against the theory that less differentiated cells are the only ones that reprogram (Eggan, et al. 2004; Eminli, et al. 2009; Hanna, et al. 2008; Hanna, et al. 2009). The great increase in reprogramming efficiency over the last years, in some cases more than 100-fold, is also not in the line of an elite model. The increase in reprogramming efficiency is due to the addition of chemicals such as VPA, AZA, 2i+LIF as well as more stringent selection. This would all argue that the number of reprogramming-susceptible cells is not fixed, but does it mean that all cells can be reprogrammed? The question was elegantly answered by a recent publication (Hanna, et al. 2009), where they took advantage of inducible so called “secondary” iPS cells (Markoulaki, et al. 2009; Wernig, et al. 2008a). Secondary iPS cells are derived from somatic cells carrying the same pattern of integration (number and location) of drug-inducible *Oct4, Sox2, Klf4* and *cMyc* viral transgenes. The reprogramming is initiated simultaneously by the addition of doxycycline to the culture medium. The cell-to-cell variation that is normally seen in transduced cultures is thus avoided. Hanna et al. demonstrated that >92% of all cells (monoclonal B-cells) could be reprogrammed over an 18-week period. The process could even be accelerated 2-fold using p53 or p21 inhibition or *Lin28* overexpression. Furthermore, they concluded that this acceleration in reprogramming was caused by an equal shortening of the overall cell cycle length. The astonishing number of reprogrammed cells clearly points to the direction that reprogramming is a stochastic process, where most likely all cells have the potential to be reprogrammed.

IPS CELLS FROM ADULT NEURAL STEM CELLS

Tissue specific stem cells, such as adult neural stem cells endogenously express pluripotency markers (*Sox2, Klf4* and *cMyc)* as well as (*SSEA-1* and AP) (Kim, et al. 2008a). They are therefore considered to be at a more advanced stage in the reprogramming process than terminally differentiated cells. Ectopic expression of *Oct4* alone, together with the endogenous expression of *Sox2, Klf4* and *cMyc* was shown to be sufficient to reprogram adult mouse and human foetal neural stem cells to a pluripotent stage (Kim, et al. 2009a; Kim, et al. 2009b). One-factor iPS cells are interesting in many aspects. First, they show that reprogramming can occur with the addition of only one factor, eliminating the need for oncogenic factors and reducing the risk for integrating tumorogenesis. Second the factor *Oct4* is sufficient and required for reprogramming both human and mouse neural stem cell, and third, the capacity to reprogram neural stem cells with only one factor might indicate that the neural stem cells are transcriptionally closer to pluripotent cells than mature fully differentiated cells.

CHEMICAL REPROGRAMMING

Human and mouse mature cells have been successfully reprogrammed with only two factors, always using *Oct4* but in combination with, *Klf4, cMyc* (Maherali and Hochedlinger 2009; Shi, et al. 2008a) or *Sox2* (Huangfu, et al. 2008b). Adult mouse neural stem cells and human foetal neural stem cells are the only cell types, that have been reprogrammed using only one factor, *Oct4* (Kim, et al. 2009a; Kim, et al. 2009b). This marks major progression in comparison to the four-factor induced reprogramming strategy. Omitting the need for any exogenous DNA either integrating or episomal is of greatest interest. This has so far only been described in one publication where they used four recombinant cell-penetrating reprogramming proteins, *Oct4, Sox2, Klf4* and *cMyc* (Zhou, et al. 2009). This later strategy still requires the addition of oncogenic factors. A pure chemical induced reprogramming strategy would be preferred, providing the possibility to circumventing the need for both exogenous DNA and oncogenic proteins. There are a few publications reporting the use of chemical instead of one or two of

original reprogramming transcription factors. The factors replaced are Sox2, Klf4 or cMyc (Huangfu, et al. 2008a; Ichida, et al. 2009; Lyssiotis, et al. 2009; Maherali and Hochedlinger 2009; Shi, et al. 2008a). However, to my knowledge, there are no publications presenting an all-chemical reprogramming strategy.

AIMS OF THE THESIS

Listed according to publication

I. To develop a new miniaturized platform for *in vitro* assays of embryonic and adult stem cells.

IIa. To develop a simple and efficient technique to transfect cells of the CNS, enabling gain- or loss-of function studies as well as fate mapping *in vivo*.

IIb. To investigate the role of cadherins in the integrity of the lateral ventricle wall.

IIIa. To reprogram neural stem cells using only three factors.

IIIb. To investigate whether 4-hydroxy tamoxifen induced translocation of cMyc can contribute to reprogramming of neural stem cells.

IV. To investigate whether adult neural stem cells can be pushed towards a reprogrammed state using only chemicals.

SUMMARY OF RESULTS

Listed according to publication

Publication I describes the development of a miniaturized platform for *in vitro* studies of stem cells. The miniaturized platform consists of 672 wells, each holding 500nL. The outer size of the platform has standard slide format and fits therefore into standard laboratory equipments such as microscopes, plate-readers or flow cytometers. The platform has a glass bottom enabling excellent optical conditions for automatic or manual analysis. Three kinds of stem cells were cultured in the platform, mouse ES cells, human ES cells and mouse adult neural stem cells. The miniaturized platform allowed culturing assays for maintenance of stem cells, both in bulk assays and clonally. Differentiation assays along the ectodermal lineage was also successfully performed.

Publication II describes the development of a rapid and efficient transfection method *in vivo* by the use of electroporation. The two major germinal regions of the CNS, the SVZ and the DG of hippocampus were successfully targeted in adult mice. The use of electrical current made it possible to direct the transfection to specific parts of the lateral wall by altering the positioning of the electrodes. The transfection resulted in efficient plasmid uptake and expression. Expressions of the transfected plasmids were detected as early as 36h post electroporation and up to 4 months post electroporation, this enables short- and long-term experiments. Simultaneous injection of three plasmids resulted in over 98% co-expression enabling the use of *in vivo* electroporation for more complex experiments. The use of cell population specific promoters enabled subpopulation specific expression.

The targeting efficiency varied with the concentration and the size of the injected plasmid, giving the highest targeting efficiency for small highly concentrated plasmids. The targeted cells were mainly ependymal cells, but also cells from the adjacent subependymal layer, such as GFAP-positive cells, Ki67-positive cells and Dcx-positive cells (for lateral ventricle wall electroporation). Furthermore, fate-mapping experiments in the SVZ demonstrated that targeted cells could give rise to neurons in the olfactory bulb. Targeting of cells in the DG of the hippocampus provided a patch of transfected cells. These cells were mature NeuN-positive neurons within the granular cell layer and immature Vimentin-positive cells in the SGZ.

Optimal conditions for plasmid uptake in the CNS was evaluated and found to be 200V and 5 pulses each lasting 50mili seconds. The passing of electrical current through the CNS of the mice did not cause any detectable damage such as burns or wounding. This was also assessed by western blot for injury response related proteins and EEG-measurements. A normal EEG pattern was detected within 2h post electroporation.

The *in vivo* electroporation method was used to assess the role of cadherins in the lateral ventricle wall. A DN-cadherin construct was electroporated into the lateral wall resulting in reduction of endogenous cadherin expression and detachment of the ependymal cells already 2 days post electroporation. This was followed by polyp-like structures protruding into the lumen of the ventricle.

Publication III is one of the first publications reporting reprogramming to pluripotency omitting exogenous *Sox2* from the original cocktail of four factors. This was accomplished by the use of neural stem cells that endogenously express *Sox2*. The neural stem cells were positive for Nestin and could be differentiated to neurons (TujI) and glial cells (GFAP). A 4-OH tamoxifen inducible cMyc ($MYCER^{TAM}$) was constructed to allow cellular localization control of cMyc. The established neural stem cells were transduction with retroviruses containing the factors *Oct4, Klf4* and *cMyc* or $MYCER^{TAM}$. The reprogramming efficiency was about 0.4% for *cMyc* and 0.001% for inducible c*Myc*. The reprogramming efficiency was not dependent on whether *Sox2* was added to the cocktail or not, demonstrating that the endogenous expression of *Sox2* was sufficient to promote factor-induced reprogramming of neural stem cells. Three iPS cell colonies were expanded and one of these was further analysed in comparison with wild type ES cells. Endogenous mRNA and protein levels of *Oct4* and *Sox2* were found to be similar to ES cells. Further analysis of the iPS cell clone revealed expression of the pluripotency markers *SSEA1*, AP and *Oct4* to the same degree as in ES cells and that they were karyotypically normal. A genome-wide expression profile on the iPS clone showed high correlation to ES cell expression, (Pearson correlation = 0.98, iPS cells vs. ES cells). The iPS cells were tested for pluripotency *in vitro* using the embryonic body assay. The iPS cells gave rise to cell types characteristic of the three germ layers. Furthermore, the iPS cells were tested *in vivo* for teratoma formation and chimera production capacity. The teratomas displayed differentiation into all germ layers and in total twelve mice were derived from blastocyst injected iPS cells. Eight

out of the twelve mice showed chimerism, 5-15% evaluated by the coat color. Neither of these animals managed to produce any offspring derived from the injected iPS cells. Moreover two female mice developed tumours, with residual viral expression of inducible *cMyc* as well as *Klf4*.

Manuscript IV intends to assess whether chemicals can be used to induce or replace endogenous *Oct4* expression in adult neural stem cells. Three chemical conditions demonstrated induction of endogenous *Oct4* expression, N^6-Phenyladenosin (OA), Bupivacaine hydrochloride (OB) and a combination of OB and the chemical Tyrphostin (OT). Endogenous expression levels were measured using quantitative RT-PCR and the highest levels of *Oct4* expression was detected in the combination of OB and OT chemicals. Induced endogenous expression levels of the pluripotency genes *Nanog* and *Rex1* were also detected following 7 days of chemical exposure. The highest levels of *Nanog* and *Rex1* were detected with the combination of OB and OT.

An unexpected result of the treatment was loss of adhesion properties displayed both in the neurosphere cultures and adherent neural stem cell cultures. However, small clusters of cells were detected at the bottom of the culture dish, three days after removal of chemicals and addition of ES cell medium.

METHODOLOGICAL CONSIDERATIONS, DISCUSSION AND CONCLUSIONS

Listed according to publication

Publication I

The miniaturized platform described in publication I was developed to function as a screening platform for stem cells. The use of the platform is intended for more industrialized scales of screening, where a large number of substances or culture conditions can be studied in parallel. The advantage of the platform is also a limitation when working in smaller scale. The high throughput of the platform is limited by the available technical devices, such as flow cytometers for single cell seeding enabling clonal assays, robots for liquid handling and detection instruments for high throughput analysis. The platform was tested in all of these instruments with good results. However, much of the work presented in the publication is performed by manual handling, coating, seeding, medium exchange and analysis. This is time consuming and far from optimal, but provides a proof-of-principle concerning culturing assays of different stem cell types.

Another disadvantage in culturing stem cells is the fast proliferation, which requires dissociation of clones every 3-7 days depending on the stem cell type. Thus, our investigation of stem cell cultures was limited to 7 days. Longer culturing assays are of course possible but require automatic devices to split the cells. This did not affect differentiation assays performed on these stem cells.

The platform provides a functional tool for large-scale screening of various stem cell types, human and mouse ES cells as well as mouse adult neural stem cells. It offers simultaneous culturing and analysis of 672 different conditions at once. The size of the wells are small enough to drastically reduce regency cost, but large enough to enable weeklong experiments. The platform allows various kinds of assays, such as clonal experiments of stem cells and differentiation experiments.

Publication II

Publication II describes the development of an *in vivo* electroporation method, complementary or in some cases even an alternative to transgenic animals or virus-

mediated delivery of constructs. The method is an extension of the previously established *in utero* electroporation technique and adult mammalian muscle electroporation. The method has several advantages. First, it is fast, requires only plasmid DNA, eliminating the need for virus or animal production. Second, several constructs can be electroporated at once, enabling complex experiments. Third, the contra-lateral side automatically provides a matched control for the experiment. Fourth, it allows directed targeting of cells or population-specific expression in various regions of the CNS.

The ventricle wall has the advantage of being adjacent to the fluid-filled ventricular system, allowing spread of the injected plasmid over larger areas and thus allowing a high targeting efficiency. This is however not the case for other regions of the CNS, such as the DG of the hippocampus, where only a compact patch is targeted. Another limitation of the restricted targeting is that only cells first in line are transfected, that is ependymal cells and few cells of the subventricular region. These are the same limitations as for virus-mediated delivery of constructs.

The electroporation method also raises some concerns when it comes to injuries. The injection of a plasmid creates an injury and a glial scar along the needle tract. This is however inevitable also when using viruses. The analysis can be performed on the contra-lateral side of the injection to ensure that any effects of the injection are avoided. The effect of current-induced injury responses is also highly relevant. Five 200V pulses are passed through the CNS of the anesthetised adult mouse. No superficial damage, such as burns, was detected and no differences in injury-related markers were identified at the protein level. EEG-recordings demonstrated that there were current-induced alterations in the theta oscillations immediately after the electroporation, but this was normalized between 30min to 2h post electroporation. Application of electrical current to the CNS is performed in humans as a therapy for affective disorders. These treatments lead to detectable changes in neurogenesis and apoptosis, in a seizure dependent manner, in experimental animals. The current level used to target cells through electroporation never reached up to the seizure inducing levels performed on humans.

Cadherins (E- and N-cadherins) were shown to play a vital role for the ependymal layer integrity. The expression of cadherins co-localized primarily with the S100β-positive ependymal cells. Lowering of endogenous cadherins using a DN-cadherin, (an N-terminal truncated version of human N-cadherin) resulted in loss of ependymal cells. This might not be too surprising, since cadherins are known to play a

vital role in adherens junction formations. The ependymal cells were found to fall off due to reduced cell attachment capacities and not to any major extent due to increased cell death caused by the treatment. A similar result was seen through expression of DN-cadherin introduced by adenovirus.

In conclusion, genetic manipulations of adult mouse neurogenic niches are possible using an *in vivo* electroporation strategy. Plasmid DNA is injected and transfected to a region of choice without detectable damage. Both loss- and gain-of-function as well as fate mapping experiments were performed and cadherins were demonstrated to play an important role in the integrity of the lateral ventricle wall.

Publication III

Publication III demonstrates that the endogenous expression level of *Sox2* in neural stem cells is sufficient for the reprogramming into pluripotent iPS cells. The addition of *Sox2* to the cocktail of three factors did not increase the overall reprogramming efficiency. Later publications came to the same conclusion, and even further reduced the number of factors needed down to only *Oct4*. Several attempts to derive iPS cells without *cMyc* were made, but none of them resulted in ES-like colonies. We speculated in the article that this could be due to the difference in the reprogramming process between fibroblasts and neural progenitor cells. Following publications have demonstrated that *cMyc* can be omitted from the reprogramming cocktail also in the case of neural stem cells, however, with reduced reprogramming efficiency. An inducible cMyc was created to control the localization of cMyc and to reduce the tumorigenic risk. This led to severe reduction in the reprogramming efficiency and to the unfortunate development of tumours in two female mice. One reason for the reduced reprogramming efficiency for inducible cMyc could be that the levels and the duration of nuclear cMyc were much decreased in comparison with non-inducible cMyc. The tumours were found to be entirely iPS cell derived and high in inducible cMyc expression. Both mice generating tumours had only weeks before delivered pups and we speculated that the effect of inducible cMyc was pregnancy related. No further investigation was made to rule out other causes, such as insertional mutagenesis caused by the viruses at the time of publication. Subsequently, studies have demonstrated that the iPS cells had a normal number of chromosomes but carried a karyotypic abnormality, that was discovered by combined binary ratio fluorescence in situ

hybridization. The iPS cells had a translocation of a part of chromosome 11 to the telomeric region of chromosome 8. To our knowledge, this translocation has never been described in relation to any tumours. However, we cannot rule out the contribution of the translocation to the tumour or that virus mediated insertional mutagenesis was the cause of the tumours.

Neural precursor cells were derived and used instead of adult neural stem cells for two main reasons. First, to efficiently generate selectable neural precursor cells by targeting with an Oct4PurΔTK construct, allowing us to select reprogrammed cells through positive puromycin selection. Second, to generate clonally derived cells at sufficient numbers to perform the experiments needed. The choice of retroviral delivery was to ensure high and long-term expression of the reprogramming factors, and efficient silencing of virus expression upon complete reprogramming.

The iPS clone demonstrated *Oct4* levels similar to ES levels. Quantitative RT-PCR was performed to ensure that the expression levels were endogenous and not proviral derived. The high endogenous expression levels of *Nanog*, which is undetectable in neural stem cell, indicates that reactivation of endogenous locus has occurred.

Out of eight colonies that were picked, three could be expanded and only one was ES-like. One explanation to the lack of expansion could be that the colonies were not fully reprogrammed, but rather representing a pre-reprogrammed state. This could perhaps have been modulated with the addition of ES medium containing MEK-inhibitor and GSK-inhibitor in addition to LIF, as shown after publication of this work (Silva, et al. 2008), in order to help pre-reprogrammed cells to reach a fully reprogrammed state. These are only speculations but it would have been interesting to investigate further. The levels of chimerism detected were fairly low, based on coat color, PCR analysis of proviral integration in various organs and the lack of germline transmission. Later studies performed on the iPS cells revealed chromosome instability. This might also provide an explanation to the lack of iPS cell derived offspring.

Manuscript IV

Manuscript IV concerns an all-chemical approach towards reprogramming of adult neural stem cells. Previous publications have shown that *Oct4* is the only factor that needs to be added to adult neural stem cells in order to create fully reprogrammed cells (Kim, et al. 2009b) and also that reprogramming factors can be substituted by chemicals, so that they reduce the need for exogenous DNA (Ichida, et al. 2009; Maherali and Hochedlinger 2009). Candidate chemicals in this manuscript were found in a large chemical screen performed on fibroblasts, in an attempt to find chemicals that could either induce or replace *Oct4* expression. Four of the candidate chemicals discovered were tested in this manuscript. They were N6-Phenyladenosin or Oct4-A (OA), Bupivacaine hydrochloride or Oct4-B (OB), Sinomenine or Oct4-S (OS) and Tyrphostin or Oct4-T (OT).

Three out of the four chemicals had an effect on endogenous *Oct4* expression levels also in adult neural stem cells measured on mRNA levels. Nevertheless, the expression levels detected were not at the same level as *Oct4* expression in ES cells. However, the mRNA analysis was performed on cell populations, not clonally. This provides an average value of the overall expression levels and a potential underestimation of the individual expression levels for some clones. mRNA levels of *Nanog* and *Rex1* were also measured to investigate whether the induced *Oct4* levels could result in re-expression of other pluripotency factors. Similar chemical conditions found to induce *Oct4* also induced *Nanog* expression and in some cases also *Rex1* expression. The detection of mRNA levels only is not enough to state that the adult neural stem cells are reprogrammed. Further analysis of the cells needs to be performed. However, the detection of endogenous expression of both *Oct4* and *Nanog* is interesting and could indicate that the adult neural stem cells are capable to respond in that direction, without the addition of exogenous DNA.

Chemicals were tested on both neurosphere cells and adherent adult neural stem cells. Expression levels of *Sox2* and *cMyc* were similar, but the level of *Klf4* was found to be higher in neurosphere cultures in comparison to adherent cultures. Adherent cultures have some advantages compared to neurosphere cultures in this situation: the number of cells in each experiment is easier to control, appearing colonies are much easier to detect, the overall number of cells generated are higher and the colonies can be expanded for longer times and even frozen down for future analysis.

The loss-of-adhesion properties detected early on in all the chemical treated cells, both free-floating and adherent, lack explanation so far. One speculation is

that they lose their N-cadherin expression and fails to upregulate E-cadherin expression.

The overall findings after addition of the candidate chemicals are interesting and potentially promising, but further experiments and more analyses needs to be performed in order to conclude whether adult neural stem cells can reprogrammed to *bona fide* iPS cells using an all-chemical approach.

In conclusion, this study aims at an all-chemical approach towards reprogramming of adult neural stem cells. Three candidate chemicals were found to induce endogenous *Oct4* expression in adult neural stem cells. The levels of *Oct4* were much lower than the expression levels detected in ES cells. However, the *Oct4* expression was sufficient to induce endogenous expression of the pluripotency genes *Nanog* and *Rex1*. These findings need to be further analysed to verify whether iPS cells can be generated from adult neural stem cells through an all-chemical strategy.

ACKNOWLEDGEMENTS

Tack Jonas för att du är den bästa tänkbara handledare en doktorand kan tänka sig!
Att doktorera i ditt labb har varit otroligt spännande, utmanande och framförallt roligt!

Ett oändligt stort tack till min familj, ni är helt underbara!

Tillslut, tack till min fantastiska Alex, utan dig vore jag ingenting!

REFERENCES

ABS Global,
1997, Calf Cloned From Bovine Cell Line. Science 277(5328): 903

Altman, J.
1969 Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. J Comp Neurol 137(4):433-57.

Altman, J., and G. D. Das
1965 Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124(3):319-35.

Amati, B., K. Alevizopoulos, and J. Vlach
1998 Myc and the cell cycle. Front Biosci 3:d250-68.

Anthony, T. E., et al.
2004 Radial glia serve as neuronal progenitors in all regions of the central nervous system. Neuron 41(6):881-90.

Aoi, T., et al.
2008 Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 321(5889):699-702.

Askanazy, M.
1907 Verh. Dtsch. Pathol. Ges. 11(39-82):39.

Bagga, S., et al.
2005 Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 122(4):553-63.

Bannerman, D. M., et al.
2004 Regional dissociations within the hippocampus--memory and anxiety. Neurosci Biobehav Rev 28(3):273-83.

Becker, T., et al.
1997 Axonal regrowth after spinal cord transection in adult zebrafish. J Comp Neurol 377(4):577-95.

Bez, A., et al.
2003 Neurosphere and neurosphere-forming cells: morphological and ultrastructural characterization. Brain Res 993(1-2):18-29.

Bhutani, N., et al.
2009 Reprogramming towards pluripotency requires AID-dependent DNA demethylation. Nature.

Biebl, M., et al.
2000 Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. Neurosci Lett 291(1):17-20.

Blau, H. M., and B. T. Blakely
1999 Plasticity of cell fate: insights from heterokaryons. Semin Cell Dev Biol 10(3):267-72.

Blelloch, R., et al.
2006 Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells 24(9):2007-13.

Boyer, L. A., et al.
2005 Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947-56.

Bradley, A., et al.

1984 Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309(5965):255-6.
Briggs, R., and T. J. King
1952 Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. Proc Natl Acad Sci U S A 38(5):455-63.
Brill, M. S., et al.
2009 Adult generation of glutamatergic olfactory bulb interneurons. Nat Neurosci 12(12):1524-33.
Brockes, J. P.
1997 Amphibian limb regeneration: rebuilding a complex structure. Science 276(5309):81-7.
Bruni, J. E.
1998 Ependymal development, proliferation, and functions: a review. Microsc Res Tech 41(1):2-13.
Bruni, J. E., M. R. Del Bigio, and R. E. Clattenburg
1985 Ependyma: normal and pathological. A review of the literature. Brain Res 356(1):1-19.
Byrne, J. A., et al.
2007 Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature 450(7169):497-502.
Cajal, Ramon y
1913 Estudios sobre la degeneración y regeneración del sistema nervioso. Madrid: Moyal.
Caldwell, M. A., et al.
2001 Growth factors regulate the survival and fate of cells derived from human neurospheres. Nat Biotechnol 19(5):475-9.
Cameron, H. A., and R. D. McKay
2001 Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 435(4):406-17.
Campbell, K. H., et al.
1996 Sheep cloned by nuclear transfer from a cultured cell line. Nature 380(6569):64-6.
Carlen, M., et al.
2009 Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. Nat Neurosci 12(3):259-67.
Chang, M. Y., et al.
2004 Properties of cortical precursor cells cultured long term are similar to those of precursors at later developmental stages. Brain Res Dev Brain Res 153(1):89-96.
Chen, C., et al.
2007 Defining embryonic stem cell identity using differentiation-related microRNAs and their potential targets. Mamm Genome 18(5):316-27.
Conti, L., et al.
2005 Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PLoS Biol 3(9):e283.
Coskun, V., et al.
2008 CD133+ neural stem cells in the ependyma of mammalian postnatal forebrain. Proc Natl Acad Sci U S A 105(3):1026-31.
Cowan, C. A., et al.
2005 Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309(5739):1369-73.
Curtis, M. A., et al.

2007 Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315(5816):1243-9.

D'Amour, K. A., and F. H. Gage
2003 Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. Proc Natl Acad Sci U S A 100 Suppl 1:11866-72.

Do, J. T., et al.
2008 Enhanced reprogramming of Xist by induced upregulation of Tsix and Dnmt3a. Stem Cells 26(11):2821-31.

Do, J. T., and H. R. Scholer
2004 Nuclei of embryonic stem cells reprogram somatic cells. Stem Cells 22(6):941-9.

Doetsch, F., and A. Alvarez-Buylla
1996 Network of tangential pathways for neuronal migration in adult mammalian brain. Proc Natl Acad Sci U S A 93(25):14895-900.

Doetsch, F., et al.
1999aSubventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97(6):703-16.

Doetsch, F., J. M. Garcia-Verdugo, and A. Alvarez-Buylla
1999b Regeneration of a germinal layer in the adult mammalian brain. Proc Natl Acad Sci U S A 96(20):11619-24.

Du, T., and P. D. Zamore
2005 microPrimer: the biogenesis and function of microRNA. Development 132(21):4645-52.

Duinsbergen, D., et al.
2008 Induced pluripotency with endogenous and inducible genes. Exp Cell Res 314(17):3255-63.

Eggan, K., et al.
2004 Mice cloned from olfactory sensory neurons. Nature 428(6978):44-9.

Eminli, S., et al.
2009 Differentiation stage determines potential of hematopoietic cells for reprogramming into induced pluripotent stem cells. Nat Genet 41(9):968-76.

Eriksson, P. S., et al.
1998 Neurogenesis in the adult human hippocampus. Nat Med 4(11):1313-7.

Esteban, M. A., et al.
2009 Vitamin C Enhances the Generation of Mouse and Human Induced Pluripotent Stem Cells. Cell Stem Cell.

Evans, M. J., and M. H. Kaufman
1981 Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154-6.

Feng, B., et al.
2009 Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. Nat Cell Biol 11(2):197-203.

Ferron, S., et al.
2004 Telomere shortening and chromosomal instability abrogates proliferation of adult but not embryonic neural stem cells. Development 131(16):4059-70.

Galli, C., et al.
2003 Pregnancy: a cloned horse born to its dam twin. Nature 424(6949):635.

Garcia, A. D., et al.
2004 GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci 7(11):1233-41.

Garthe, A., J. Behr, and G. Kempermann

2009 Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One 4(5):e5464.

Goldman, J. E.
1995 Lineage, migration, and fate determination of postnatal subventricular zone cells in the mammalian CNS. J Neurooncol 24(1):61-4.

Golmohammadi, M. G., et al.
2008 Comparative analysis of the frequency and distribution of stem and progenitor cells in the adult mouse brain. Stem Cells 26(4):979-87.

Gratzner, H. G.
1982 Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science 218(4571):474-5.

Gritti, A., et al.
2002 Multipotent neural stem cells reside into the rostral extension and olfactory bulb of adult rodents. J Neurosci 22(2):437-45.

Gurdon, J. B.
1962 Adult frogs derived from the nuclei of single somatic cells. Dev Biol 4:256-73.

Gurdon, J. B., and V. Uehlinger
1966 "Fertile" intestine nuclei. Nature 210(5042):1240-1.

Hack, M. A., et al.
2004 Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. Mol Cell Neurosci 25(4):664-78.

Hanna, J., et al.
2008 Direct reprogramming of terminally differentiated mature B lymphocytes to pluripotency. Cell 133(2):250-64.

Hanna, J., et al.
2009 Direct cell reprogramming is a stochastic process amenable to acceleration. Nature 462(7273):595-601.

Harris, H.
1967 The reactivation of the red cell nucleus. J Cell Sci 2(1):23-32.

Hastings, N. B., and E. Gould
1999 Rapid extension of axons into the CA3 region by adult-generated granule cells. J Comp Neurol 413(1):146-54.

Henze, D. A., N. N. Urban, and G. Barrionuevo
2000 The multifarious hippocampal mossy fiber pathway: a review. Neuroscience 98(3):407-27.

Hitoshi, S., et al.
2002 Neural stem cell lineages are regionally specified, but not committed, within distinct compartments of the developing brain. Development 129(1):233-44.

Hong, H., et al.
2009 Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. Nature 460(7259):1132-5.

Huangfu, D., et al.
2008a Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat Biotechnol 26(7):795-7.

Huangfu, D., et al.
2008b Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat Biotechnol 26(11):1269-75.

Ichida, J. K., et al.
2009 A small-molecule inhibitor of tgf-Beta signaling replaces sox2 in reprogramming by inducing nanog. Cell Stem Cell 5(5):491-503.

Imayoshi, I., et al.
2008 Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11(10):1153-61.
Imura, T., H. I. Kornblum, and M. V. Sofroniew
2003 The predominant neural stem cell isolated from postnatal and adult forebrain but not early embryonic forebrain expresses GFAP. J Neurosci 23(7):2824-32.
Irvin, D. K., et al.
2003 Extrinsic and intrinsic factors governing cell fate in cortical progenitor cultures. Dev Neurosci 25(2-4):162-72.
Ivey, K. N., et al.
2008 MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell 2(3):219-29.
Jackson, E. L., and A. Alvarez-Buylla
2008 Characterization of adult neural stem cells and their relation to brain tumors. Cells Tissues Organs 188(1-2):212-24.
Johns, P. R., and S. S. Easter, Jr.
1977 Growth of the adult goldfish eye. II. Increase in retinal cell number. J Comp Neurol 176(3):331-41.
Judson, R. L., et al.
2009 Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol 27(5):459-61.
Kaji, K., et al.
2009 Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458(7239):771-5.
Kaneko, Y., et al.
2000 Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22(1-2):139-53.
Kaplan, M. S.
1981 Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol 195(2):323-38.
Kee, N., et al.
2007 Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10(3):355-62.
Kempermann, G., et al.
2003 Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130(2):391-9.
Kim, J. B., et al.
2009a Direct reprogramming of human neural stem cells by OCT4. Nature 461(7264):649-3.
Kim, J. B., et al.
2009b Oct4-induced pluripotency in adult neural stem cells. Cell 136(3):411-9.
Kim, J. B., et al.
2008a Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. Nature 454(7204):646-50.
Kim, J., et al.
2008b An extended transcriptional network for pluripotency of embryonic stem cells. Cell 132(6):1049-61.
Kornack, D. R., and P. Rakic
1999 Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A 96(10):5768-73.
Kubicek, S., et al.

2007 Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. Mol Cell 25(3):473-81.
Kuhn, H. G., H. Dickinson-Anson, and F. H. Gage
1996 Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16(6):2027-33.
Laywell, E. D., et al.
2000 Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci U S A 97(25):13883-8.
Lee, B. C., et al.
2005 Dogs cloned from adult somatic cells. Nature 436(7051):641.
Lee, N. S., et al.
2008 miR-302b maintains "stemness" of human embryonal carcinoma cells by post-transcriptional regulation of Cyclin D2 expression. Biochem Biophys Res Commun 377(2):434-40.
Lee, T. I., et al.
2006 Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125(2):301-13.
Lendahl, U., L. B. Zimmerman, and R. D. McKay
1990 CNS stem cells express a new class of intermediate filament protein. Cell 60(4):585-95.
Li, H., et al.
2009 The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 460(7259):1136-9.
Lin, S. L., et al.
2008 Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. RNA 14(10):2115-24.
Loh, Y. H., et al.
2006 The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38(4):431-40.
Loh, Y. H., et al.
2007 Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21(20):2545-57.
Lois, C., and A. Alvarez-Buylla
1993 Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A 90(5):2074-7.
Lois, C., and A. Alvarez-Buylla
1994 Long-distance neuronal migration in the adult mammalian brain. Science 264(5162):1145-8.
Luo, J., et al.
2008 Subventricular zone-mediated ependyma repair in the adult mammalian brain. J Neurosci 28(14):3804-13.
Luskin, M. B.
1993 Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11(1):173-89.
Lyssiotis, C. A., et al.
2009 Reprogramming of murine fibroblasts to induced pluripotent stem cells with chemical complementation of Klf4. Proc Natl Acad Sci U S A 106(22):8912-7.
Magavi, S. S., et al.
2005 Adult-born and preexisting olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses in vivo. J Neurosci 25(46):10729-39.

Maherali, N., and K. Hochedlinger
2009 Tgfbeta signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. Curr Biol 19(20):1718-23.
Maherali, N., et al.
2007 Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 1(1):55-70.
Markoulaki, S., et al.
2009 Transgenic mice with defined combinations of drug-inducible reprogramming factors. Nat Biotechnol 27(2):169-71.
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(12):7634-8.
Marzesco, A. M., et al.
2005 Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. J Cell Sci 118(Pt 13):2849-58.
Masui, S., et al.
2007 Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9(6):625-35.
McCulloch, E. A., and J. E. Till
1960 The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. Radiat Res 13:115-25.
Merkle, F. T., and A. Alvarez-Buylla
2006 Neural stem cells in mammalian development. Curr Opin Cell Biol 18(6):704-9.
Merkle, F. T., Z. Mirzadeh, and A. Alvarez-Buylla
2007 Mosaic organization of neural stem cells in the adult brain. Science 317(5836):381-4.
Merkle, F. T., et al.
2004 Radial glia give rise to adult neural stem cells in the subventricular zone. Proc Natl Acad Sci U S A 101(50):17528-32.
Mikkelsen, T. S., et al.
2008 Dissecting direct reprogramming through integrative genomic analysis. Nature 454(7200):49-55.
Miller, R. A., and F. H. Ruddle
1976 Pluripotent teratocarcinoma-thymus somatic cell hybrids. Cell 9(1):45-55.
Mirzadeh, Z., et al.
2010 Cilia organize ependymal planar polarity. J Neurosci 30(7):2600-10.
Mirzadeh, Z., et al.
2008 Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell 3(3):265-78.
Morshead, C. M., et al.
2002 Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. Nat Med 8(3):268-73.
Nakagawa, M., et al.
2008 Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 26(1):101-6.
Newman, M. A., J. M. Thomson, and S. M. Hammond
2008 Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. RNA 14(8):1539-49.

Nottebohm, F., et al.
1990 Song learning in birds: the relation between perception and production. Philos Trans R Soc Lond B Biol Sci 329(1253):115-24.
Okita, K., T. Ichisaka, and S. Yamanaka
2007 Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313-7.
Okita, K., et al.
2008 Generation of mouse induced pluripotent stem cells without viral vectors. Science 322(5903):949-53.
Oppenheim, R. W.
1991 Cell death during development of the nervous system. Annu Rev Neurosci 14:453-501.
Ostenfeld, T., et al.
2002 Regional specification of rodent and human neurospheres. Brain Res Dev Brain Res 134(1-2):43-55.
Palmer, T. D., A. R. Willhoite, and F. H. Gage
2000 Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425(4):479-94.
Parmar, M., et al.
2003 Phenotypic and molecular identity of cells in the adult subventricular zone. in vivo and after expansion in vitro. Mol Cell Neurosci 24(3):741-52.
Parmar, M., et al.
2002 Regional specification of neurosphere cultures derived from subregions of the embryonic telencephalon. Mol Cell Neurosci 21(4):645-56.
Polejaeva, I. A., et al.
2000 Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407(6800):86-90.
Pollard, S. M., et al.
2006 Adherent neural stem (NS) cells from fetal and adult forebrain. Cereb Cortex 16 Suppl 1:i112-20.
Pomerantz, J., and H. M. Blau
2004 Nuclear reprogramming: a key to stem cell function in regenerative medicine. Nat Cell Biol 6(9):810-6.
Rakic, P.
1985aDNA synthesis and cell division in the adult primate brain. Ann N Y Acad Sci 457:193-211.
Rakic, P.
1985b Limits of neurogenesis in primates. Science 227(4690):1054-6.
Reynolds, B. A., W. Tetzlaff, and S. Weiss
1992 A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. J Neurosci 12(11):4565-74.
Reynolds, B. A., and S. Weiss
1992 Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052):1707-10.
Reynolds, B. A. et al.
1996 Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 175(1):1-13.
Richards, L. J., T. J. Kilpatrick, and P. F. Bartlett
1992 De novo generation of neuronal cells from the adult mouse brain. Proc Natl Acad Sci U S A 89(18):8591-5.
Rousselot, P., N. Heintz, and F. Nottebohm

1997 Expression of brain lipid binding protein in the brain of the adult canary and its implications for adult neurogenesis. J Comp Neurol 385(3):415-26.

Sanai, N., et al.
2004 Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427(6976):740-4.

Santa-Olalla, J., et al.
2003 The in vivo positional identity gene expression code is not preserved in neural stem cells grown in culture. Eur J Neurosci 18(5):1073-84.

Sawamoto, K., et al.
2006 New neurons follow the flow of cerebrospinal fluid in the adult brain. Science 311(5761):629-32.

Seaberg, R. M., and D. van der Kooy
2003 Stem and progenitor cells: the premature desertion of rigorous definitions. Trends Neurosci 26(3):125-31.

Seri, B., et al.
2004 Cell types, lineage, and architecture of the germinal zone in the adult dentate gyrus. J Comp Neurol 478(4):359-78.

Seri, B., et al.
2001 Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21(18):7153-60.

Shen, Q., et al.
2008 Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell 3(3):289-300.

Shi, Y., et al.
2008aInduction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 3(5):568-74.

Shi, Y., et al.
2008b A combined chemical and genetic approach for the generation of induced pluripotent stem cells. Cell Stem Cell 2(6):525-8.

Shin, T., et al.
2002 A cat cloned by nuclear transplantation. Nature 415(6874):859.

Silva, J., et al.
2008 Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 6(10):e253.

Sinkkonen, L., et al.
2008 MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15(3):259-67.

Spassky, N., et al.
2005 Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. J Neurosci 25(1):10-8.

Spemann, Hans
1938 Embryonic Development and Induction. New Haven, CT (Yale Univ. Press).

Sridharan, R., et al.
2009 Role of the murine reprogramming factors in the induction of pluripotency. Cell 136(2):364-77.

Stadtfeld, M., K. Brennand, and K. Hochedlinger
2008aReprogramming of pancreatic beta cells into induced pluripotent stem cells. Curr Biol 18(12):890-4.

Stadtfeld, M., et al.

2008b Induced pluripotent stem cells generated without viral integration. Science 322(5903):945-9.
Suh, H., et al.
2007 In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. Cell Stem Cell 1(5):515-28.
Suslov, O. N., et al.
2002 Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. Proc Natl Acad Sci U S A 99(22):14506-11.
Tada, M., et al.
2003 Pluripotency of reprogrammed somatic genomes in embryonic stem hybrid cells. Dev Dyn 227(4):504-10.
Tada, M., et al.
2001 Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11(19):1553-8.
Takahashi, K., et al.
2007 Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861-72.
Takahashi, K., and S. Yamanaka
2006 Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-76.
Tavazoie, M., et al.
2008 A specialized vascular niche for adult neural stem cells. Cell Stem Cell 3(3):279-88.
Thomas, E. D., et al.
1957 Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257(11):491-6.
Thomson, J. A., et al.
1998 Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145-7.
Thomson, J. A., et al.
1995 Isolation of a primate embryonic stem cell line. Proc Natl Acad Sci U S A 92(17):7844-8.
Uchida, N., et al.
2000 Direct isolation of human central nervous system stem cells. Proc Natl Acad Sci U S A 97(26):14720-5.
van Praag, H., et al.
2002 Functional neurogenesis in the adult hippocampus. Nature 415(6875):1030-4.
Viswanathan, S. R., G. Q. Daley, and R. I. Gregory
2008 Selective blockade of microRNA processing by Lin28. Science 320(5872):97-100.
Vukicevic, V., et al.
2010 Genetic instability and diminished differentiation capacity in long-term cultured mouse neurosphere cells. Mech Ageing Dev 131(2):124-32.
Wakayama, T., et al.
1998 Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394(6691):369-74.
Wang, Y., et al.
2008 Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. Nat Genet 40(12):1478-83.
Warner-Schmidt, J. L., and R. S. Duman

2006 Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. Hippocampus 16(3):239-49.

Weigmann, A., et al.
1997 Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. Proc Natl Acad Sci U S A 94(23):12425-30.

Weiss, S., et al.
1996 Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J Neurosci 16(23):7599-609.

Wernig, M., et al.
2008aA drug-inducible transgenic system for direct reprogramming of multiple somatic cell types. Nat Biotechnol 26(8):916-24.

Wernig, M., et al.
2008b c-Myc is dispensable for direct reprogramming of mouse fibroblasts. Cell Stem Cell 2(1):10-2.

Wernig, M., et al.
2007 In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448(7151):318-24.

Winner, B., et al.
2002 Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb. Eur J Neurosci 16(9):1681-9.

Yoshida, Y., et al.
2009 Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell 5(3):237-41.

Yu, J., et al.
2006 Human embryonic stem cells reprogram myeloid precursors following cell-cell fusion. Stem Cells 24(1):168-76.

Yu, J., et al.
2007 Induced pluripotent stem cell lines derived from human somatic cells. Science 318(5858):1917-20.

Zhao, C., W. Deng, and F. H. Gage
2008 Mechanisms and functional implications of adult neurogenesis. Cell 132(4):645-60.

Zhao, C., et al.
2006 Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26(1):3-11.

Zhao, Y., and D. Srivastava
2007 A developmental view of microRNA function. Trends Biochem Sci 32(4):189-97.

Zhou, H., et al.
2009 Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4(5):381-4.

Zhou, W., and C. R. Freed
2009 Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27(11):2667-74.