View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Publications from Karolinska Institutet

From DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
Karolinska Institutet, Stockholm, Sweden

IONIC MODULATORS OF STEM CELL STATE

Anna Omelyanenko

Stockholm 2016

All previously published papers were reproduced with permission from the publisher.
Published by Karolinska Institutet.
Printed by AJ E-print AB
© Anna Omelyanenko, 2016
ISBN 978-91-7676-286-8

Ionic Modulators of Stem Cell State

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Anna Omelyanenko

Principal Supervisor:
Assistant Professor Michael Andäng
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Pharmacology

Co-supervisor(s):
Dr. Helena Johard
Karolinska Institutet
Department of Physiology and Pharmacology
Division of Pharmacology

Dr. Gabriella Lundkvist
Karolinska Institutet
Department of Neuroscience

Prof. Per Uhlen
Karolinska Institutet
Department of Biochemistry and Biophysics
Division of Molecular Neurobiology

Opponent:
Professor Hans-Georg Kuhn
University of Gothenburg
Department of Clinical Neuroscience

Examination Board:
Assistant Professor Anna Falk
Karolinska Institutet
Department of Neuroscience

Professor Finn Hallböök
Uppsala University
Department of Neuroscience
Division of Developmental Neuroscience

Associate Professor Erik Sundström
Karolinska Institutet
Department of Neurobiology, Care Sciences and Society
Division of Neurodegeneration

To new beginnings

ABSTRACT

Tissue generation during development and maintenance throughout life relies on the proliferation and sequential specification of a group of cells. Stem cells are defined by the properties of self-renewal (division to produce daughter cells equipotent to the mother) and differentiation (division where at least one daughter is of a more restricted potential). In adult systems, two additional states - quiescence, a dormant state of infrequent proliferation, and activation, a state of increased proliferation, are described. Regulation of these states is a key determinant of health and fitness on a tissue and organism level, as it ensures proper development and regeneration. The aim of this thesis is to investigate how another key system at the cellular level – regulation of ion availability – can modulate cell states of embryonic and adult stem cells.

In **paper I** the effect of lithium chloride (LiCl) on juvenile mouse neural stem progenitor cells (NSPCs) from the subgranular zone (SGZ) of the hippocampus was investigated. Under maintenance conditions, treatment with LiCl increased NSPC proliferation, reducing the fraction of cells in G0/G1. Pre-treatment of NSPCs with LiCl prior to ionizing radiation (IR) exposure reduced DNA damage response activation, and attenuated the IR-induced G1 block, restoring proliferation, although cell death was not reduced.

In **paper II** the effect of ZD7288, a specific blocker of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, on mouse embryonic stem cell (ESCs) was examined. The blocker attenuated proliferation by extending G1 and S phases. This did not compromise pluripotency, but facilitated spontaneous serum-induced differentiation while reducing the efficiency of directed differentiation towards the neuronal lineage.

In **paper III** expression of HCN family channels and effects of their inhibition in adult NSPCs were described. *Hcn2* and *Hcn3* are expressed throughout the NSPC hierarchy, but only functional in S and G2/M phases. HCN inhibition or knockdown attenuated proliferation due to a reversible G0/G1 accumulation which was accompanied by alterations in activation marker expression, metabolism, and the molecular clock network. A small molecular agonist of Rev-erb-α, a clock component, recapitulated the proliferative effects. HCN inhibition-induced G0/G1 block was shown to have a protective effect during IR exposure of juvenile mice, reducing apoptosis and maintaining proliferation.

In conclusion, lithium, which is proposed to inhibit a number of enzymes by replacing magnesium as a cofactor, and HCN currents, which are involved in regulation of the electrochemical state of the cell, were shown to modulate stem cell state. This suggests that further investigation of these and other ionic modulators is warranted, both for therapy development and in the interests of basic science.

LIST OF SCIENTIFIC PAPERS

I. Zanni G*, Di Martino E*, **Omelyanenko A**, Andäng M, Delle U, Elmroth K, Blomgren K. Lithium increases proliferation of hippocampal neural stem/progenitor cells and rescues irradiation-induced cell cycle arrest in vitro. *Oncotarget*, 2015, 6(35):37083-97

II. **Omelyanenko A**, Sekyrova P, Andäng M. ZD7288, a blocker of the HCN channel family, increases doubling time of mouse embryonic stem cells and modulates differentiation outcomes in a context-dependent manner. *SpringerPlus*, 2016, 5:41

III. Johard H*, **Omelyanenko A***, Gao F*, Zilberter M, Youssef R, Harisankar A, Trantirek L, Walfridsson J, Linnarsson S, Lundkvist G, Harkany T, Blomgren K, Andäng M. Hyperpolarization-activated cyclic nucleotide-gated channels modulate active proliferation and metabolism, and maintain molecular clock oscillations in adult mouse neural stem progenitor cells. *Manuscript*

*denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

Akt	Protein kinase B
ALP	Alkaline phosphatase
APC/C	Anaphase-promoting complex
Ara-C	Arabinofuranosyl cytidine
ASCL1	Achaete-scute homolog 1
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia mutated Rad3-related
BMAL1	Aryl hydrocarbon receptor nuclear translocator-like protein 1
BMP4	Bone morphogenic protein 4
BMPT1	Bisphosphate 3'-nucleotidase 1
BrdU	5-bromo-2'-deoxyuridine
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Cdc20	Cell-division cycle protein 20
CDK	Cyclin-dependent kinase
CHD7	Chromodomain-helicase-DNA-binding protein 7
Chk1	Checkpoint kinase 1
CKI	Cell-cycle dependent kinase inhibitory protein
CNS	Central nervous system
Cry1	Cryptochrome 1
CsCl	Cesium chloride

DNA	Deoxyribonucleic acid
DNMT3a	DNA (cytosine-5)-methyltransferase 3A
E3.5	Embryonic day 3.5
EdU	5-Ethynyl-2′-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
giCSC	Human glioma-initiating cancer stem cell
GO	Gene Ontology
GSK3	Glycogen synthase kinase 3
HCN	Hyperpolarization-activated cyclic nucleotide-gated channel
hfNSC	Human fetal neural stem cell
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
IMPase	Inositol monophosphatase
IP3	Inositol-1,4,5 triphosphate
IPPase	Inositol polyphosphate 1-phosphotase
iPSC	Induced pluripotent stem cell
IR	Ionizing radiation

K^+	Potassium
K_i	Constant of inhibition
LiCl	Lithium chloride
LIF	Leukemia inhibitory factor
Mad2	Mitotic arrest deficient 2
MAP2	Microtubule-associated protein 2
MEK	Mitogen-activated protein kinase
Mg^{2+}	Magnesium
Msi1	RNA-binding protein Musashi homolog 1
Na^+	Sodium
NANOG	Nanog
NCX	Sodium-calcium exchanger
NFIX	Nuclear factor 1 X-type
Nr1d1	Nuclear receptor subfamily 1 group D member 1
NSPC	Neural stem progenitor cell
OCT3/4	Octamer-binding transcription factor 4
OLIG2	Oligodendrocyte transcription factor 2
Pax6	Paired box protein Pax-6
PER2	Period 2
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
qRT-PCR	Quantitative real-time polymerase chain reaction
Rb	Retinoblastoma protein

REST	RE1-Silencing Transcription factor
RNA	Ribonucleic acid
SGZ	Subgranular zone
shRNA	Short hairpin RNA
siRNA	Short silencing RNA
SMIT1	Sodium/myo-inositol cotransporter 1
SOX2	SRY (sex determining region Y)-box 2
SSEA1	Stage-specific embryonic antigen 1
SVZ	Subventricular zone
TEA	Tetraethylammonium
TLX	Nuclear receptor subfamily 2 group E member 1
γ-H2AX	Phosphorylated Histone H2A

1 INTRODUCTION

1.1 THE STEM CELL PARADIGM

We all start life as a single cell and end it as a multitude. From a single fertilised egg the many cell types found in the adult body emerge. This phenomenon represents a fundamental difference between uni- and multicellular organisms, yet the molecular mechanisms deciding cellular birth and death are remarkably similar from yeast to human. While the lifecycle of even a unicellular eukaryote, such as yeast, already allows access to a number of cell fates (division, mating, apoptosis), the increased complexity of multicellular life requires the introduction of additional fate states, at least in some cells. Today's answer to this conceptual challenge is introduction of a category of cells with greater developmental potential than mature somatic cells – the so called stem cells. These are cells possessing the two properties essential for making development (embryonic stem cells) and maintenance (adult stem cells) of multicellular life as we observe it possible: self-renewal and differentiation[1].

Self-renewal is the property of dividing to give rise to two daughter cells of which at least one has the same developmental potential as the original cell. This ensures the possibility of maintaining the stem cells population (in case of asymmetric division) while increasing cell number, or even expanding the stem cell pool (in case of symmetric division to two cells equipotent to the mother cell). Although not every stem cell will undergo self-renewal every time, it is a cardinal property of stem cells that under the right conditions they may do so. This property is essentially no different from regular division of adult cells – a fibroblast that will always be a fibroblast may divide to give rise to two fibroblasts of the same potential, which also can divide to give rise to fibroblasts. What makes self-renewal different from simple division is its tie to the second cardinal property of stem cells: differentiation.

Differentiation is the property of having the potential to give rise to cells with a developmental potential distinct from, and more restricted than that of the original cell. This allows first the creation of the germ layers and distinct lineages, and finally the generation of terminally differentiated cells, either capable of division or that have exited the cell cycle. These cells can then develop the distinct cell physiology that allows them to be best suited for their role in the tissue and the organism.

1.1.1 Stem cells in regeneration: quiescent, active, and differentiated

While embryonic stem cell lineages are generally a transient fate state, in organs with substantial cell turnover, life-long self-renewal and differentiation of a progenitor population

is the primary source of new cells. This allows mature cells to be functionalized without compromising tissue turn over, since when the cells reach the end of their life cycle they are replaced through differentiation of newly divided cells higher up along the differentiation hierarchy. Studies of the blood system in adult mice were instrumental both in the initial definition and development of the stem cell paradigm. Many credit James Till and Earnest McCullough with the "discovery" of stem cells during their work on hematopoiesis[2]. Based on their findings that the blood system of a bone marrow-ablated host could be fully reconstituted, even in the long term, by some cells from the bone marrow of a donor, both the defining properties and the functional definition of stem cells in the blood system were formulated. Serving as a prototype in stem cell biology, the hematopoietic system is still one of the best understood and certainly most clinically relevant adult stem cell systems.

Till and McCullough also reported equipotent cells that were long- or short-term repopulating based on the length of time during which they were able to sustain hematopoiesis. The long-term repopulating cells were found to be label retaining, indicating low division frequency. This combination of high potency and low division frequency represents the state now commonly referred to as "quiescence". This concept is applicable in many adult stem cell systems and the quiescent and activated (proliferative) states are associated with expression or repression of particular genes. Well studied in the blood system, these genes, and even hematopoietic stem cell (HSC) properties, vary depending on the timing and location of hematopoiesis, indicating that stem cell state is an emergent property of intrinsic factors and environmental influence.

Environmental factors (secreted and physical) that a stem cell experiences are often collectively called "the niche". The niche is known to play an important role in regulating all aspects of stem cell behaviour. Both secreted and physical niche factors have been reported to regulate stem cell pool size[3], and inherent proliferative properties. As an example, long-term repopulating HSCs in the liver, the site of fetal hematopoiesis in mammals, are more proliferative and express different surface markers than they do in the bone marrow, the site of adult hematopoiesis[4]. Interestingly, the location of the HSC niche varies not only through development but also among species: from kidney in fish, to bone marrow in birds. Perhaps most intriguingly, the HSC niche seasonally migrates between the bone marrow and the liver in some frogs[4,5]. This diversity may reflect different functional requirements for the niche (e. g. accessibility of external cues, protection from damage), understanding which may be instrumental to mobilizing endogenous or using adult stem cells for therapy.

1.2 ADULT NEUROGENESIS

The brain and the central nervous system (CNS) were long believed to exclusively be populated by non-proliferative cells, as there is no obvious ongoing turnover or need for regeneration, unlike in the blood system[6]. This was questioned extensively in the 1960's, when Altman and coworkers published a number of papers showing proliferation in the adult brain and providing evidence of neurogenesis in the mouse and other mammals[7-9]. Evidence of adult neurogenesis in humans only became available in 1998, when Eriksson and coworkers published their work identifying dividing cells in the brains of cancer patients[10]. At present, the two neurogenic niches most extensively studied in mammalian brains are the subgranular zone (SGZ) of the hippocampus, and the subventricular zone (SVZ) (see Figure 1) of the lateral ventricle, with ongoing controversy regarding the existence of additional niches[11]. Although proliferative activity is more abundant in the SVZ in the mouse[12], both niches are populated by cells which undergo division and give rise to new neurons. The SGZ produces granule neurons and astrocytes for the dentate gyrus, whereas SVZ gives rise to oligodendrocytes and to neuroblasts that migrate along the rostral migratory stream, becoming olfactory bulb interneurons[13,14]. The dynamics of neurogenesis have also been investigated, and the number of dividing cells as well as newly born neurons was shown to decrease with age both in mice[15,16] and humans[17].

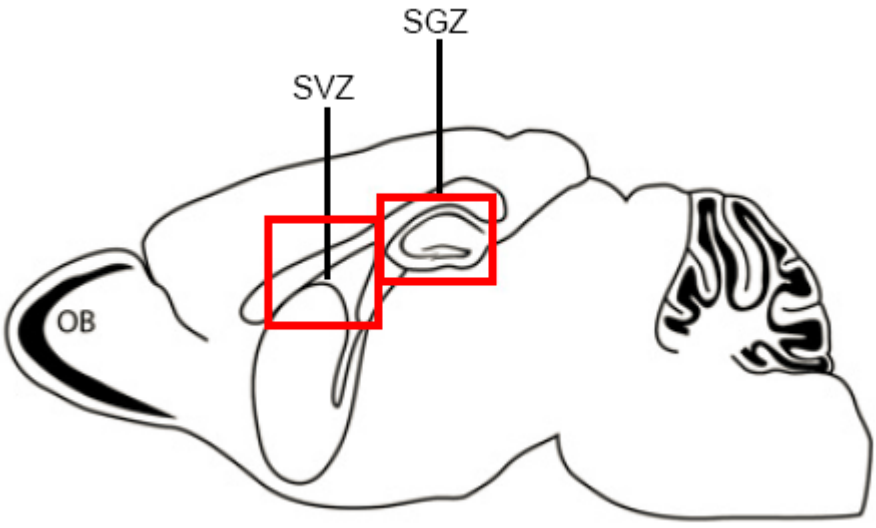

Figure 1: Neurogenic zones in the mouse brain. Modified from Genes and Development, 26 (10), Hsieh J, Orchestrating transcriptional control of adult neurogenesis, 1010-1021 (2012) under the Creative Commons license.

Unlike in other adult tissues, the main role of the proliferative progenitor compartment in the brain appears to be facilitation of plasticity, rather than tissue maintenance and replacement of exhausted cells. On a cellular level new-born neurons in the adult brain possess electrical

properties unique from those of mature neurons and can thus code information differently[18]. On a tissue level, newly generated neurons form new synapses, modifying existing neural circuitry[19]. When looking at the level of cognitive function and behaviour in the mouse, neurogenesis contributes to both short- and long-term memory. Olfactory bulb neurogenesis is believed to be important for short-term memory and olfactory learning[20,21], while neurogenesis in the hippocampus is important for long-term memory formation and pattern recognition[20,22]. Adult neurogenesis has also been suggested to be involved in mood regulation and hippocampal neurogenesis was shown to be necessary for function of a number of antidepressants[23,24]. Intriguingly, neurogenesis was also shown to be increased in response to damage, such as due to epileptic seizures[25] and stroke[26], in line with a more conventional role of tissue repair and regeneration for the neurogenic pool.

With the homeostatic and potentially injury repair functions of adult neurogenesis in mind, a number of clinical interventions are possible. Transplantation or activation of endogenous neural precursors into an injured brain is a potential strategy. This has been tested in Parkinsonian patients who received fetal cell transplants[27]. Additionally, modulation of proliferation to affect mood disorders may in fact be current practice already, as a number of antidepressant therapies are hypothesized to function through inducing proliferation[28,29]. In patients experiencing loss of neurogenic potential an alternative clinical strategy is preservation or enhancement of the proliferative pool to improve cognitive outcomes. This is particularly relevant for when the injury is a side effect of therapy, such as what is seen in treatment of pediatric brain cancers[30]. Adults who had suffered from brain cancer as children were found to have reduced neurocognitive abilities, with degree of impairment proportional to dose of radiation received[31]. Given that postnatal neurogenesis is highest at younger ages, it has been proposed that irreversible damage to the proliferating progenitor pool may underlie the impairments observed later in life[32]. Strategies to address this have included stimulating neurogenesis after the event[33], as well as neuroprotective neoadjuvant treatments[34], including the well-known mood stabilizer, lithium[35].

1.2.1 Stem cell hierarchy in the adult mouse brain

Neural cells originate from the ectodermal layer of the embryo, which forms the neurotube made up by neuroepithelial cells, a symmetrically and rapidly dividing population of early neuronal progenitors. Neuroepithelial cells are further specified to become radial glia cells, which continue to divide symmetrically, self-renewing until the onset of neurogenesis. As neurogenesis commences radial glia cells start to divide asymmetrically giving rise to

intermediate progenitor cells that go on to further differentiate to neurons. Following neurogenesis, gliogenesis is initiated, and when that nears its close, radial glia cells start to divide symmetrically, exhausting their pool[36,37]. Neurogenesis in gyrencephalic species (e.g. sheep, primates) involves additional intermediate cell types, necessary to build up the many cortical layers that are not found in the most commonly studied lissencephalic animals (mouse) discussed here[38].

It is not entirely clear which neurogenic cells in the embryonic developmental cascade give rise to adult neural stem progenitor cells (NSPCs). It has been suggested that SGZ NSPCs are generated late during gestation in the ventral hippocampus and migrate to their eventual location by birth[39], whereas SVZ NSPCs are generated *in situ* and simply remain quiescent until the end of neurogenesis[40]. Postnatally, the neurogenic cascade in the SVZ (see Figure 2A) starts with the mostly quiescent, radial glia-like type B cells, located close to the ventricular wall projecting a cilium into the ventricle. These cells express the GFAP and stem cell marker SOX2 and persist over a long time. The transit-amplifying type C cells occupy the same niche, but are further away from the ventricle, proliferate much more, and give rise to type A cells, which are the migrating neuroblasts destined to become neurons[41]. In the

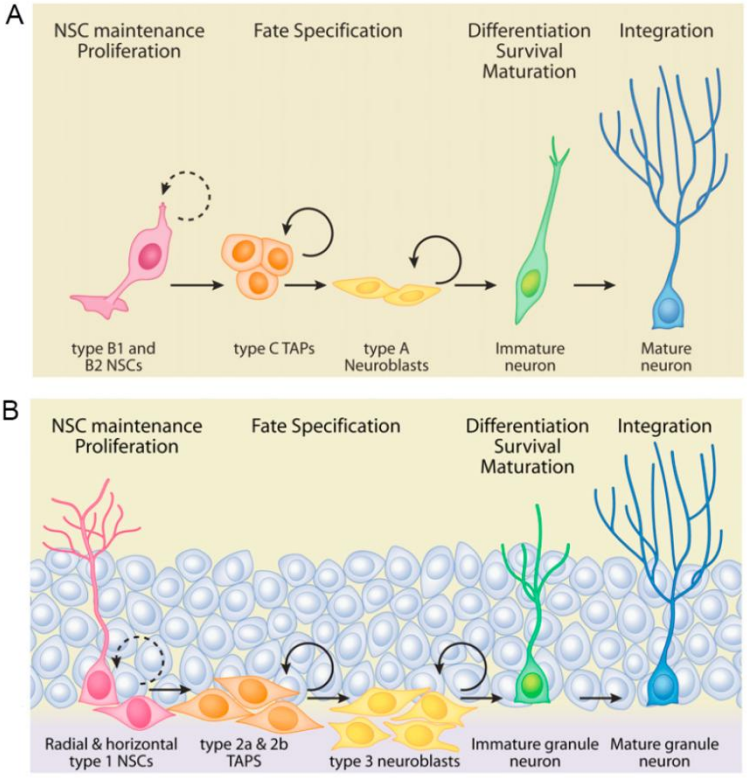

Figure 2: (A) In the SVZ NSPC hierarchy starts with type B cells, transit amplifying type C cells, and type A neuroblasts. (B) in the SGZ radial type I are followed by transit amplifying type II cells and type 3 neuroblasts. Modified from Genes and Development, 26 (10), Hsieh J, Orchestrating transcriptional control of adult neurogenesis, 1010-1021 (2012) under the Creative Commons license.

SGZ (see Figure 2B), type I cells represent the main progenitor population and, similar to radial glia during embryonic neurogenesis, have projections spanning the entire granule cell layer. Like type B cells in the SVZ, they express GFAP as well as SOX2. There are also type

II cells that are less similar to radial glia than type I cells, due to lack of GFAP expression and a shortened morphology, but that still express SOX2. There is significant debate regarding their identity in relation to type I cells, with some arguing that they are more restricted progeny, while others believe the two cell types represent interconvertible pools[19,42]. Finally, the SGZ also contain intermediate progenitors that undergo symmetrical division to give rise to neural cells without further proliferative potential[43]. Currently, identification of the various NSPC types relies on their position and morphology *in vivo*, as well as expression of a number of characteristic markers, of which SOX2, GFAP, Prominin1, and Nestin are associated with the most potent cell type (type B and type I).

SVZ and SGZ NSPCs are usually isolated for *in vitro* culture as neurospheres, by dissecting out the relevant anatomical brain region, dissociating the tissue and growing the resulting cell in a defined media supplemented with fibroblast growth factor (FGF) and epidermal growth factor (EGF), which have been shown to support NSPC expansion. Bulk cultures from both neurogenic regions have been shown to exhibit stem cell properties, as they are self-renewing and multipotent, giving rise to neurons, astrocytes and oligodendrocytes *in vitro*[44,45]. *In vitro* differentiation of clonal SVZ-derived cells initially indicated bipotentiality only, with only neuronal and glial progeny observed[46], and more recently, it has been suggested that clonally SVZ NSPC can only give rise to neurons[47]. This is in line with *in vivo* reports of single lineage differentiation of SVZ precursors, which also suggested their rapid exhaustion once proliferation is initiated[48]. Similar controversy applies to the SGZ, where there are reports of both exhaustion[49] and cycling between active proliferation and quiescence[50]. These disparate findings leading to the use of the term "stem progenitor cell", as they bring up the questions of whether NSPCs are *bona fide* stem cells very tightly controlled by their niche, or represent separate progenitor populations with limited stem cell potential. However, work showing that *in vitro* cultured SGZ NSPCs give rise to SVZ-specific cell types *in vivo*[51] when transplanted into the SVZ argues for the former.

1.2.2 Cell cycle in NSPCs

Cell cycle control in NSPCs involves the canonical cell cycle machinery and follows the usual order of G1-S-G2-M, but with a prominent G0, especially *in vivo*. While G0 is the quiescent phase when cells are neither undergoing not preparing for division, G1 and G2 are growth phases when the cells are preparing for DNA synthesis and for cell division, respectively. The entire process is regulated by sequential activation of cyclin-dependent

kinases (CDKs) and transcription of specific genes, and involves three checkpoints, at G1/S transition, in G2, and in M phase (see Figure 3).

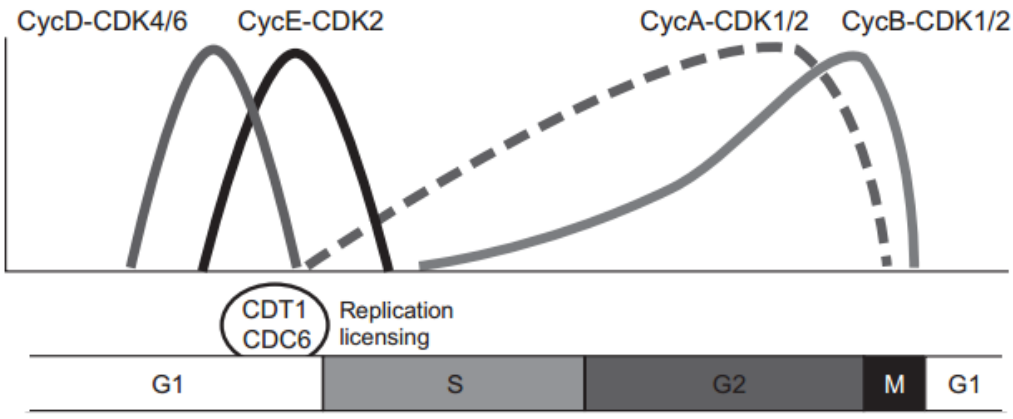

Figure 3: Cyclical activity of cyclins and CDKs regulates progression of somatic cells through the cell cycle. Reprinted from Current topics in developmental biology, 104, Tsubouchi T, Fisher A. Reprogramming and the Pluripotent Stem Cell Cycle. 223-241 (2013) with permission from Elsevier.

Cyclins D (D1/D2/D3) are regulated by mitogen signalling and together with CDK4 and 6 regulate G1 progression by phosphorylating the retinoblastoma protein (Rb), releasing it from binding E2F. Once sufficient E2F is available, cyclins E and A are transcribed and the cell cycle proceeds when cyclin E-CDK2 phosphorylation of Rb reaches critical levels, passing the so called "restriction point" and committing the cell to division. CDK2 then cooperates with cyclin A to phosphorylate DNA synthesis machinery as the S-phase progresses. With the end of S-phase, CDK2 is replaced with CDK1, which cooperates with cyclin A and the G2/M cyclin B to phosphorylate G2/M targets. Once the M-phase is complete and the cell has divided, the cell cycle machinery is reset to the start and cyclin D starts to build up in response to mitogen signalling, if cycling is continuous. The activity of CDKs is regulated not only by the availability of cyclins, but also by their subcellular localization, degradation, and inhibition by cyclin-dependent kinase inhibitor proteins (CKIs). CKIs include INK4 proteins (p15, p16, p18, and p19) that regulate CDK4 and 6 activity and G1 progression, and the Cip/Kip family (p21,p27,p57) that regulate CDK activity in both G1 and G2, and DNA synthesis directly (in the case of p21)[52,53].

Expression of CKIs is most notably regulated by p53, which is upregulated in response to DNA damage and is the main effector in the G1 DNA damage checkpoint. In response to DNA damage during G1, p53 upregulates CKI expression, preventing G1/S progression and inducing G1 arrest. It can also play a role in G2 DNA damage response, where p53-induced p21 expression can inhibit CDK1. G2/M progression is abrogated if DNA damage is present

even in the absence of p53, due to the action of checkpoint kinases 1 and 2 (Chk1 and Chk2). Cell cycle stalling in response to DNA damage allows repair or induction of apoptosis to safeguard genomic integrity in the daughter cells, and is regulated by the ataxia telangiectasia mutated (ATM) and Rad3-related (ATR) proteins. The final checkpoint is the mitotic spindle checkpoint in the M-phase, which ensures correct chromosomal segregation during division. If the mitotic spindle and chromosomes are not correctly assembled, the cells stall due to mitotic arrest deficient 2 (Mad2)-mediated inhibition of cell-division cycle protein 20 (Cdc20), resulting in inhibition of the anaphase-promoting complex (APC/C), which would otherwise be driving the cell cycle forward by ubiquitination and degradation of cell cycle machinery[52,54].

1.2.3 Quiescence and activation in NSPCs

The more intriguing and unique aspect of NSPC cell cycle regulation is the regulation of G0/G1 balance. Early studies examining neurogenesis in the adult brain using anti-mitotic drug identified that the majority of NSPCs *in vivo* are quiescent in the G0 cell cycle phase[41]. Recent reports are suggesting that although not mitotically active, quiescence is not a passive state and presence of signalling is necessary for its maintenance[55]. WNT signalling has been shown to regulate quiescence[56], as does γ-aminobutyric acid (GABA) signalling through the $GABA_A$ receptor, the absence of which resulted in NSPC reactivation following irradiation injury[57,58]. Prominin1, a hallmark of NSPCs, is expressed in primary cilia, known for acting as signalling centres, and is also characteristic of quiescence[59]. In fact, Prominin1 expression coupled with lack of epidermal growth factor receptor (EGFR) expression is commonly used to identify quiescent cells[60], with the absence of EGFR highlighting their low dependence on mitogens.

In line with this, quiescent cells have been shown to have altered metabolic requirements[61], be resistant to mitochondrial perturbations[62] and have gene expression signatures consistent with glycolytic metabolism[63]. A number of cell-intrinsic parameters, such as expression of lineage-related transcription factors (SOX2, REST[64], OLIG2[65], ASCL1[66]), the nuclear receptor TLX [67], circadian regulator PER2[68], epigenetic modifiers (NFIX[69], DNMT3a[70]), and cell cycle machinery (CHD7[71], p27[72]) have been linked to regulation of the stem cell pool and the balance between quiescence and activation. Niche factors such as growth factor signaling through FGF2 and bone morphogenic protein 4 (BMP4)[73] and vascular contact[74] have also been implicated in maintenance of quiescence and activation. Unfortunately, the mechanisms

of interconversion between the two pools and, in particular of quiescence induction, are still poorly understood, highlighting the need for further study.

1.3 EMBRYONIC STEM CELLS

Mouse embryonic stem cells (ESCs) were first isolated and cultured in 1981 from the inner cell mass of the preimplantation blastocyst at embryonic day 3.5 (E3.5), or the epiblast at E4.5, and represent cells of the late pre-implantation blastocyst[75] (see Figure 4). A similar cell type can be derived from early human pre-implantation blastocysts that are the by-product of *in vitro* fertilization, although these cells represent a developmentally distinct cell type. In normal development, ESCs give rise to the three embryonic germ layers – mesoderm, endoderm, and ectoderm, making all the adult tissues of embryonic origin. *In vitro*, they can be maintained indefinitely (as they express telomerase and are not subject to telomere shortening) without losing their stem cell properties, provided they are cultured under permissive conditions.

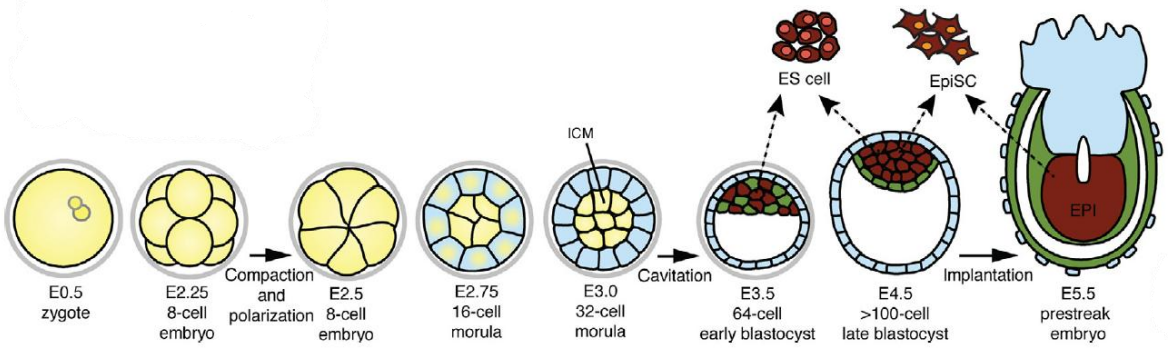

Figure 4: Early development of the mouse. Embryonic stem cells are derived from and correspond to E3.5 or E4.5 the pre-implantation blastocyst. Reprinted from Current topics in developmental biology, 107, Posfai E, Tam OH, Rossant J. Mechanisms of pluripotency in vivo and in vitro. 1-37 (2014) with permission from Elsevier.

The ability of ESCs to be cultured long term is remarkable not only because they are not transformed, but also because unlike adult stem cells, which persist for many years *in vivo*, they usually represent a very transient state in mouse development. An exception exists in diapause – a facultative stalling of mouse embryo development at an about 130 cell stage induced by metabolic restriction, such as lactation of the mother[76]. At this time, metabolic processes such as glycolysis, in the cells of the blastocyst are downregulated[77], and autophagy has been proposed to take place in some cells to generate nutrients for the others[78]. The process is proposed to be regulated by microRNAs[79] and results in G0/G1 stalling[80] and major alterations in cell metabolism[76], resembling quiescence of adult stem cells. Recent work comparing transcriptional profiles of diapaused and developing blastocysts has in fact

identified E4.5 as the active stage most similar to that seen in the diapaused epiblast[81], suggesting that *in vitro* cultured ESCs should also be capable of reaching the diapaused state, but the conditions necessary for reaching it are only just beginning to be described.

ESC are fascinating for many reasons, not the least of which being their extensive proliferative capacity and ability to generate most mature cell types, given the proper inductive cues. As with many cell types, identification of conditions permissive of efficient *in vitro* maintenance and expansion is critical for allowing both basic and applied research using ESCs. Generation of knockout mice, which has become a cornerstone of biological research, has been based on ESC technology for many years now and is one example of the usefulness of ESC as tools. This process has been significantly facilitated by the development of defined culture methods, which use small molecule inhibitors to generate uniform populations of undifferentiated ESCs[82]. However, the final goal remains to develop this cell type for regenerative medicine. A decade ago a breakthrough in stem cell biology was made by the team of Shinya Yamanaka, who developed the technology for reverting mature cells to the ESC stage by induction of a set of key genes linked to ESC identity[83], drawing this goal closer. However, clinical use of ESC-based technology will still require, at the very least, efficient and well-defined protocols for stem cell differentiation as well as culture, and adaption of them to human cells.

1.3.1 Stemness and differentiation in embryonic stem cells

Embryonic stem cell state is characterized by self-renewal and the ability to differentiate to all somatic cell types (pluripotency). As such, the gold-standard functional test to determine stem cell capacity in ESCs is aggregation with or injection into a developing blastocyst, which should result in chimeric embryos with ESC contribution to all germ layers. The key to ESC identity lies in the expression of the core pluripotency transcription factors: OCT3/4, SOX2, and NANOG. Expression of these genes endows ESCs with their characteristic properties: clonogenicity (ability of a single cell to give rise to a colony) and pluripotency (ability to give rise to cells of all embryonic germ layers). Insufficient expression of these genes, on the other hand, results in ESC differentiation even under maintenance conditions, and in the case of OCT3/4, even overexpression perturbs pluripotency[84]. These transcription factors function by regulating their own and each other's expression, and cooperatively bind peripheral targets that are also involved in pluripotency maintenance, making the network even more robust. They also bind the promoters and repress the expression of differentiation inducers, such as GATA6 which drives the first *in vivo* differentiation event – the formation

of the primitive endoderm[85]. Conclusive proof of this is the ability of the core pluripotency factors to induce the ESC state in mature cells when overexpressed during the process of reprogramming, giving rise to induced pluripotent stem cells (iPSCs)[83]. This technology has been extended to human cells[86], and has initiated discussions of comprehensive human leukocyte antigen (HLA)-based donor cell line biobanking initiatives that would provide nation-wide coverage and near-universal access to future stem-cell based therapies with minimal immunological complications[87].

Efficient maintenance of the pluripotent state *in vitro* was traditionally achieved by culture in media supplemented with leukemia inhibitory factor (LIF)[88] and serum (with BMP4 believed to be the key molecule)[89], that have more recently been replaced by two small molecule inhibitors: one targeting mitogen-activated protein kinase (MEK) and the other targeting glycogen synthase kinase 3 (GSK3)[82]. The role of MEK inhibition in this culture protocol is to inhibit FGF signalling, which is responsible for initiation of differentiation already at the level of the inner cell mass, where it is essential for giving rise to the differentiated hypoblast along with the pluripotent epiblast[90]. FGF signalling is endogenous to ESCs, which produce FGF4 and express the FGF receptor, and the knockout of *Fgf4* results in impaired differentiation to both ectoderm and mesoderm lineages[91], suggesting that it plays a role in creating the heterogeneity necessary to allow exit from pluripotency and induction of lineage specification. The role of GSK3 inhibition is less clear, although the majority of its effect is believed to be related to activation of WNT signalling. Activation of WNT signalling through genetic perturbation of GSK3 phosphorylation-mediated β-catenin degradation showed a dose dependent inhibition of differentiation[92], although other effects of GSK3 inhibition, such as increase in colony forming ability and c-Myc expression were not recapitulated[82].

Major progress in defining protocols for ESC differentiation has also been made. Whereas differentiation in general is easily achieved through withdrawal of maintenance factors (LIF), induction of particular cell identity is often a lengthy process of multistage differentiation, in many cases closely mimicking development and using complex components, such as stromal cells, which are not compatible with eventual clinical use[93]. Significant progress is being made, and the development of a clinically-compatible differentiation protocol for retinal epithelium[94] has resulted in one of the first ever ESC-derived cell products entering clinical trials[95]. However, many differentiation protocols still rely on modulation of kinase or receptor activity with often costly peptides and proteins, often of non-human origin, although efforts are being made to address this[96].

1.3.2 The unique ESC cell cycle and self-renewal

Given the speed of proliferation necessary to accommodate the growth seen in the first days of development, it is not surprising that the architecture of ESC cell cycle is drastically different from that of mature cells. *In vivo*, cell cycle length at the developmental stages corresponding to ESCs has been estimated at 9.1-11.5 hours[97], while ESC doubling times under standard culture conditions vary between 8 and 12 hours[98,99], although they have been reported to increase to over 30 hours in serum-free culture[99]. In comparison, many mature cells do not proliferate, and generation times for commonly cultured mature cells, such as mouse embryonic fibroblasts, are reported to be around 19 hours[100]. The observed shorter generation time in ESCs is due to shortening of both G1 and G2 growth phases, with ~60% of cell cycle spent in S-phase, as opposed to ~16-20% of cycling time devoted to S-phase in mature cell types[98].

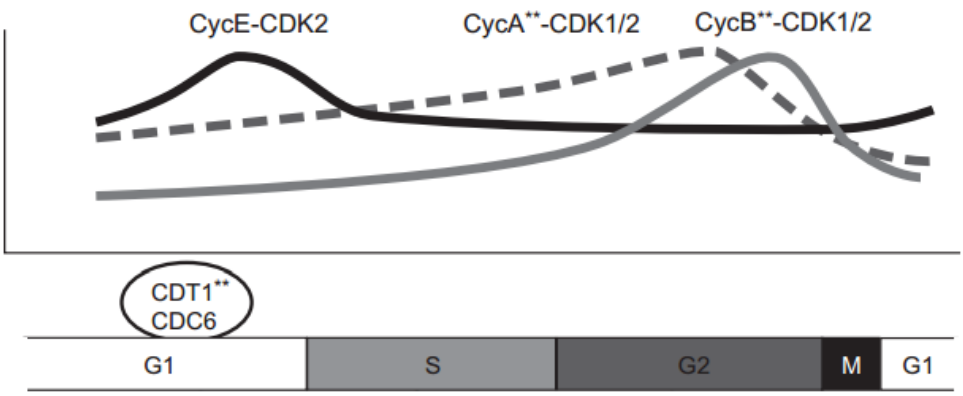

Figure 5: ESCs have a unique cell cycle characterized by constitutive activity of cycling and CDKs. Reprinted from Current topics in developmental biology, 104, Tsubouchi T, Fisher A. Reprogramming and the Pluripotent Stem Cell Cycle. 223-241 (2013) with permission from Elsevier.

This is possible due to radically altered activity of cell-cycle machinery and absence of checkpoints. In somatic cells, progression through the cell cycle is tightly regulated to ensure conditions are right for the next phase of the cell cycle to occur. As described above (see Figure 3), this is coordinated by successive activation of CDKs by their cyclin partners, which are sequentially expressed and promptly ubiquitinated and degraded, ensuring kinase activity is restricted to the proper cell cycle phase[52]. In ESCs this oscillatory behaviour is lacking (see Figure 5), with constitutive CDK2, cyclin A and cyclin E activity[98], geminin expression, which blocks DNA replication under normal conditions[101], and attenuated APC/C oscillations[102]. The usual checkpoints that are present in the cell cycle of somatic cells are also absent. The restriction point is absent from ESC, where Rb is in a phosphorylated state throughout the cell cycle[103]. Consistent with this, serum starvation does not result in cell cycle

arrest[104] indicating that mitogen signals are dispensable for ESC proliferation. p16 fails to inhibit the CDK6-CycD3 complex[105] further indicating a lack of a G1/S checkpoint. Induction of checkpoints associated with DNA damage and resulting in p53-induced cell cycle arrest, leads instead to loss of pluripotency, as translocation of p53 to the nucleus downregulates Nanog expression in ESCs[106]. Similarly, activation of the mitotic checkpoint, which is functional in ESCs, does not result in apoptosis as in somatic cells[107], a G2/M accumulation and impaired apoptosis were also seen upon CDK1 inhibition in human ESCs[108].

The role of this altered cell cycle architecture in pluripotency maintenance is debated, but the prevailing view is that a link exists. G1 length has been most discussed in this context, as differentiation is accompanied by G1 lengthening[109], while reprogramming to a pluripotent state both requires and results in G1 shortening[110,111]. Additionally, findings that differentiation factors are preferentially expressed in G1[112], and the G1/S inhibitor p27 plays a role in repressing *Sox2* during differentiation[113] further link cell cycle control in G1 and pluripotency maintenance. In complete contradiction, it has also been reported that extension of G1 by exogenous expression of p21 and p27 did not compromise pluripotency under maintenance or differentiation conditions[114], although the same authors later reported improved differentiation when G1 lengthening was induced by culture in low serum conditions[115].

1.4 THE ROLE OF IONS AND THEIR REGULATION IN EUKARYOTIC CELLS

Whether *in vivo* or *in vitro*, cells exist in an aqueous environment which allows diffusion of nutrients, signalling molecules, and wastes to and from the cell, thus sustaining cellular metabolism. While the cell membrane is permeable to water, it is not permeable to many of the molecules and ions found in the interstitial fluid. In fact, concentrations of many ions and metabolites inside and outside of the cell are unequal, often differing by multiple orders of magnitude[116]. Sodium (Na^+) and potassium (K^+) are the most differentially distributed intracellular ions, each with an over 10-fold difference in cytoplasmic and external concentrations. This gradient is used to drive nutrient transport[117], regulate cell volume[118,119], and in signalling, by allowing rapid changes in the cell's electrical properties. Whether directly or through their downstream targets, Na^+ and K^+ currents influence multiple aspects of cellular physiology including cell division[120] and differentiation[121].

In addition to Na^+ and K^+, concentrations of other metal ions are also tightly controlled in the cell. Calcium (Ca^{2+}) can directly influence protein function by inducing conformational

changes upon binding, and is used as a messenger and as a cofactor in processes such as vesicular transport[122]. It has been well established as a regulator of differentiation[123], apoptosis[124], and neuronal firing[125]. The temporal dynamics of fluctuations in Ca^{2+} concentration, and not just the absolute concentration, have also been shown to have a regulator role.

Somewhat less well-understood are the regulatory functions of metals such as magnesium (Mg^{2+}), iron (Fe^{3+}), zinc (Zn^{2+}), copper (Cu^{2+}), and manganese (Mn^{2+}). Their concentrations are tightly regulated in the cell and they are known to be essential cofactors for essential cellular processes, such as adenosine triphosphate (ATP) utilization (Mg^{2+} [126]), ATP production in the mitochondria (Fe^{3+} [127]), detoxification (Cu^{2+}, Zn^{2+}, and Mn^{2+} [128]), and transcriptional regulation (Zn^{2+} [129]). In fact, presence of exogenous ions, such as lithium or lead, can have significant effects on development and cell physiology due to competitive inhibition of enzymes through replacements of their endogenous ionic cofactors. Despite their clear importance, study of these ions is complicated by difficulties in detection and lack of specificity for any eventual clinical applications. Although little is known about the regulation of these ions, a number of successful attempts at modulating intracellular ion concentrations to achieve cell fate effects by varying cell media compositions have been made [130,131], reflecting that the role of ions in cellular physiology is becoming increasingly appreciated.

1.4.1 Ion channels in proliferation and differentiation

Ion channel activity has been suggested to regulate proliferation already in the 1950's, when it was observed that non-proliferative cells had lower resting membrane potential than proliferative cells[132] and membrane potential varied during cell cycle progression[133]. This was further supported by the findings of Cone that hyperpolarization of dividing cells induced arrest[134], while depolarization of even post-mitotic neurons induced their proliferation[135]. Since then ion channels, in particular K^+ channels, have been linked to cell cycle progression, both due to their cell-cycle phase-specific expression and function[136,137], and the ability to manipulate proliferation via ion channel inhibition[138-140]. Ca^{2+} channels[141] and chloride channels[142] have also been implicated in cell cycle progression in multiple cell types, as has the Na^+-K^+ ATPase, which is proposed to be responsible for G1 hyperpolarization[143].

Ion channel control of stem cell proliferation has also been shown. While it may not be as surprising for NSPCs, which although not electrically active mature to be electrically active cells, it is even the case for ESCs. Regulation of ESC proliferation by ligand-gated ion

channels has previously been reported[144], with activation of the $GABA_A$ receptor carrying a chloride current resulting in accumulation of cells in the S phase. Similarly, it induced quiescence in NSPCs[145]. Block of L- and T-type Ca^{2+} channels was shown to attenuate ESC proliferation, with T-type channel block resulting in an accumulation of cells in G1 and G2/M cell cycle phases and differentiation[146]. L-type channel block caused a reduction in BrdU incorporation and cell numbers[147], which is compatible with an induction of differentiation reported for these channels[148]. Similarly, block of tetraethylammonium (TEA)-sensitive K^+ channels induced an accumulation of ESCs in G0/G1 and compromised pluripotency[149], while block of the hyperpolarization-activated cyclic nucleotide-gated (HCN) K^+ channel family resulted in S-phase accumulation of ESCs with uncharacterized effects on stem cell fate[150].

Recent publications have suggested that modulation of cellular electric properties can be an instructive factor in stem cell differentiation: optogenetic stimulation improved neural and neuronal differentiation of ESCs[151], activation of Ca^{2+}-activated potassium channels resulted in enrichment in cardiac pacemaker cells[152], and block of L-type Ca^{2+} channels, known to have an instructive effects on NSPC proliferation and differentiation[153], was shown to restrict ESC differentiation to non-mesodermal lineages[148]. Curiously, when L-type Ca^{2+} channels were blocked with a different compound, differentiation to cardiomyocytes, a mesodermal lineage, was improved[154], underlining the importance of further characterization of ion current effects on stem cell fate.

1.4.2 Lithium

Lithium is a metal that is already well established in the clinic. First introduced by John Cade for the treatment of manic patients in 1949[155], it has been a cornerstone of therapy and the target of extensive study, as deciphering its mechanism of action could be key to understanding psychosis. Its effects are however not limited to behaviour. Patients treated with lithium and responding to treatment were shown to have increased grey matter volume in the prefrontal cortex, which was not present in the non-responders[156]. In line with this, lithium enhanced proliferation of hippocampal neural progenitor cells in the mouse both under homeostatic[157,158] and radiation injury conditions[35]. Outside the brain, lithium treatment is known to increase neutrophil and reticulocyte counts[159], and has been shown to affect osteoblastic differentiation *in vitro*[160]. Interestingly, while reportedly only resulting in a mild increase in heart defects in humans[161,162], lithium can severely dysregulate embryonic

development, resulting in excessive endomesoderm formation in *Xenopus laevis*[163], and defective patterning in zebrafish and mouse embryos[164,165].

Despite many known effects and many proposed modes of action with experimental support, there is no consensus on which molecular pathway is responsible for mediating lithium action. The most discussed pathways are the inositol and GSK3β pathways. Lithium is reported to inhibit inositol monophosphatase (IMPase) with an inhibitory constant (concentration achieving 50% inhibition, K_i) of 0.8mM[166] and inositol polyphosphate 1-phosphotase (IPPase) with K_i=0.3mM[167], two enzymes critical in inositol metabolism, by replacing their cofactor Mg^{2+}. This results in reduced availability of the second messenger inositol-1,4,5 triphosphate (IP3) which is a major mediator of extracellular signals and lies upstream of protein kinase C (PKC). Alternatively, lithium has been reported to inhibit the transcription of the sodium/myo-inositol cotransporter 1 (SMIT1), a Na^+ coupled inositol transporter[168,169], also resulting in lower inositol availability. The fact that levels of inositol *in vivo* are not affected at therapeutic lithium concentrations argues against this as the major pathway through which lithium acts. In the GSK3β pathway, lithium inhibits GSK3 β, also by competing with magnesium[170], resulting in accumulation of β-catenin and dysregulation of WNT signalling, which is a key regulator of multiple cellular processes, including patterning during development and cell fate. Conflicting reports regarding behaviour of GSK3β knockout mice and the fact that lithium-induced mouse embryogenesis defects were not mediated by WNT signalling argues against GSK3β-WNT axis as the major effector of lithium action.

Some less commonly discussed targets of lithium have also been described, many in metabolic pathways. Bisphosphate 3'-nucleotidase 1 (BMPT1), an enzyme in the sulfate metabolism pathway which compromised protein synthesis when knocked out[171], is inhibited by lithium with K_i of 0.153 mM[172]. In the glycolysis and glycogenesis pathway, fructose 1,6-bisphosphatase[173] has been suggested to be inhibited, while glucose 1,6-biphosphate synthase[171] enzymatic activity was greatly slowed down by lithium. In line with this, earlier work showed that rate constants for reactions catalyzed by lithium-bound phosphoglucomutase was $4x10^8$ times slower than when the enzyme is bound to its usual cofactor magnesium[174]. In cell systems, treatment with lithium has been linked to inhibited glycogen synthesis in astrocytes[175], and long term lithium treatment *in vivo* resulted in reduced glycogen content in rat livers[176] and perturbed brain metabolite concentrations when examined over the 8 hours post injection[177].

1.4.3 Hyperpolarization-activated cyclic nucleotide-gated (HCN) family channels

Electrochemical changes on a cellular level are essential for cardiac pacemaking, muscle contraction, and neural processes. This is accomplished by the concerted action of gated ion channels, which taking advantage of the electrochemical gradients of some ion species existing between the cytoplasm and interstitial fluid, facilitate rapid and drastic changes in the electrical properties of cells. The heart is perhaps the best known example of this. Opening of voltage-gated sodium channels causes rapid Na^+ influx and cell depolarization, which deactivates the channels and activates other voltage gated channels sensitive to depolarization. Influx of Ca^{2+} and efflux of K^+ through these channels restore membrane potential to below activating voltage of the Na^+ channels that initiated the event, giving time for Na^+-Ca^{2+} exchanger (NCX) and the Na^+-K^+ ATPase to restore the ion balance. The current that then drives partial depolarization allowing the entire event to happen again, is the "funny" current (I_f, also known as I_h) carried by HCN4, one of the members of the HCN channels family[178].

Activation at hyperpolarized potentials is a unique feature of this channel family, which consists of four channel isoforms, activating at different voltages and differentially modulated by cyclic adenosine monophosphate (cAMP)[179]. The channels are permeable to Na^+ and K^+ and to a much lesser degree to Ca^{2+}, but generally conduct inward Na^+ currents due to the electrochemical gradient of the ion. The four isoforms have different tissue distribution, with HCN1 and HCN2, which have the fastest kinetics, expressed in the CNS and the heart, while HCN3 and HCN4 are expressed in selected regions of the heart and CNS, as well as in other organs, such as the lung, liver, and muscle[180]. Interestingly, the channels assemble into tetramers, which can be composed of multiple isoforms, giving rise to a wide range of conductance and activation properties. Channel function is also modulated by trafficking[181-183] , auxiliary subunits[184,185], phosphatidylinositol 4,5-bisphosphate (PIP2)[186], and G-protein coupled receptors[187].

In addition to cardiac rhythm generation, HCN function is implicated in many neuronal functions, ranging from mood regulation to regulation of circadian pacemaking and learning[188]. In non-excitatory cells, a pro-apoptotic effect of HCN2 has been described[189], and the channels have been reported to regulate the proliferation of ESCs[150]. The channels can be blocked by cesium chloride (CsCl), and due to significant interest in modulation of HCNs in epilepsy and cardiac arrhythmias, a number of small molecule inhibitors have been developed. ZD7288 is one of the most specific and commonly used HCN inhibitors[190,191],

although this has recently been put into question[192]. An elegant way of targeting the brain while avoiding cardiac effects is being explored by developing drugs targeting the interaction of HCNs with their auxiliary subunit TRIP8b[193] which is not expressed in the heart.

2 AIMS

The aim of this thesis was to investigate the action of ionic modulators on stem cell state. More specifically, the following three sub-aims were defined:

I. For lithium chloride, an ionic modulator with well-investigated non-ionic downstream effector pathways, to investigate the proliferative and anti- apoptotic effects on *in vitro* cultured juvenile mouse neural stem progenitor cells in the context of ionizing radiation.

II. For ZD7288, an inhibitor of the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family, to investigate the effects on stem cell state in a well-defined, robust, *in vitro* stem cell system (mouse embryonic stem cells).

III. To investigate effects of HCN inhibition on the proliferative state of mouse neural stem progenitor cells and identify potential downstream effector pathways underlying cell state effects.

3 MATERIALS AND METHODS

Please refer to the materials and methods sections of the included papers for detailed descriptions of experimental procedures, techniques, and reagents used.

4 RESULTS AND DISCUSSION

4.1 PAPER I: LITHIUM INCREASES PROLIFERATION OF HIPPOCAMPAL NEURAL STEM PROGENITOR CELLS AND RESCUES IRRADIATION-INDUCED CELL CYCLE ARREST *IN VITRO*

Cancer therapy involving irradiation of brain tissue in juvenile patients is associated with reduced cognitive performance later in life. One of the mechanisms proposed to mediate this effect is irreparable damage to the neural stem progenitor cells (NSPCs) in the developing brain, which are then not able to sustain proper development. Lithium chloride (LiCl), an antipsychotic drug with a long history of use in the clinic has been suggested to be a promising candidate for neuroprotection. This paper investigates the effects of lithium on mouse NSPCs *in vitro,* focusing on the cell physiology and outcomes following drug treatment and ionizing radiation (IR) exposure. To reflect the clinically relevant population, juvenile (post-natal day 8) mice were used for NSPC preparation. The SGZ, which is the site responsible for cognitive development in humans and likely affected in cancer patients, was chosen as the anatomical site from which NSPCs were isolated.

4.1.1 Lithium has a concentration-dependent effect on NSPC proliferation

To characterise the effects of LiCl on NSPCs, cells were adapted to *in vitro* culture by neurosphere formation for 4 days, following which they were dissociated to a single cell suspension and exposed to 0,1, or 3 mM LiCl in standard media.

Analysis of sphere size 24 and 48 hours following treatment initiation found increased sphere volume in 3 mM LiCl treated cultures as compared to control at both time points (increases of ≈3.7 fold and ≈3.2 fold, respectively), reflecting larger cell numbers. 1 mM LiCl treated cultures also showed a trend toward increased sphere volume. While this could be due to altered rates of cell death, levels of both apoptosis and necrosis were found to be similar in 3 mM LiCl treated and control cells. To investigate possible cell cycle effects, cell cycle distribution of treated and control cells was examined by flow cytometry analysis of DNA content at 12, 24, 48, 72, and 96 hours post treatment initiation. While there was no effect at 12 hours, the G1 fraction was reduced while the S fraction was increased in the cultures treated with 3 mM LiCl at all later time points. The G2/M fraction in LiCl treated cells was reduced compared to control at 24 hours, but increased at 48 hours following treatment. Similar trends were seen in 1 mM LiCl treated cultures. S-phase findings were confirmed by increased bromdeoxiuridin (BrdU) incorporation at 48 hours. Unaltered frequencies of cells

staining positive for glial (GFAP) and neuronal (MAP2) markers indicate that LiCl treatment was not associated with differentiation.

These findings support the previous reports that lithium increases proliferation of NSPCs, where GSK3β inhibition causing activation of WNT signalling was implicated in the response. They are also in line with reports of lithium-induced proliferation in other cell types. The observed changes in the G1 fraction may reflect altered cell cycle dynamics and a shorter G1, activation of previously quiescent cells, or some combination of these two effects.

4.1.2 Lithium protects NSPCs from irradiation, recruits them into proliferation following IR-induced G1 arrest and reduces DNA damage, but does not affect apoptosis.

To investigate potential neuroprotective effects of LiCl, NSPCs grown *in vitro* were treated with 0, 1, or 3 mM LiCl for 12 hours, following which cultures were exposed to 0 or 3.5 Gy of IR and allowed to form spheres in the same medium. IR treated cells formed smaller spheres than sham irradiated cells, with effects of radiation clear at all time points analysed (up to 72 hours) and significantly more drastic in the cells that were not pre-treated with lithium. While sphere volume in non-treated irradiated cells was ≈7.5 fold smaller than in sham irradiated cells, the reduction was only ≈2 fold in the cells that were pre-treated with 3 mM LiCl. Similar effects were observed at 24 hours and with lower (1 mM) LiCl concentration.

BrdU incorporation rates indicated that proliferation after irradiation remained higher in the 3mM LiCl treated than untreated cells, although it was lower than in 3mM LiCl treated sham irradiated cells. Interestingly, the proliferative differences were more pronounced in the irradiated cultures due to very low proliferation in the non-LiCl treated group. In fact, irradiation induced a significant G1 and G2 accumulation in both pre-treated and non-treated culture at the expense of the S-phase fractions. LiCl-treated cells recovered quicker, with more cells entering S than in the control cultures already at 24 hours after irradiation. To test if reduced DNA damage could underlie the recovery, levels of γ-H2AX staining, a marker of DNA damage, were examined 30 min after irradiation in treated and control cells. 3 mM LiCl treated cells were found to have on average 13 % lower staining intensity, suggesting a more limited extent of DNA damage. Finally, effect of treatment on cell death was examined. Staining for Annexin-V, a marker of apoptosis, and quantifying the subG1 DNA fraction indicated that LiCl treatment did not affect apoptosis or necrotic cell death.

Although LiCl treatment did not prevent IR-induced cell death, as was suggested previously[194], it did reduce IR-induced DNA damage, or at least the extent of response. This did not abolish, but rather attenuated the G1 accumulation seen after irradiation in the LiCl pretreated cells, which returned to proliferation faster than the non-pretreated cells. It is possible that this is due to lower rates of DNA damage, faster DNA repair, or more worryingly, leaky DNA damage checkpoints. It is interesting that the G2/M fraction in the LiCl pretreated cells remained elevated for up to 72 hours following irradiation. A potential explanation of this could be differential activation of DNA damage pathways in cells at different stages of the cells cycle, or impaired clearing of γ-H2AX foci due to reduced protein kinase B (Akt) activation[195] following lithium effects on the inositol pathway.

In summary, we found that LiCl induced NSPC proliferation under homeostatic conditions, and attenuated IR-induced DNA damage response and G0/G1 stalling. Although treatment did not reduce levels of cell death, cell numbers in treated cultures were increased due to increased proliferation.

4.2 PAPER II: ZD7288, A BLOCKER OF THE HCN CHANNEL FAMILY, INCREASES DOUBLING TIME OF MOUSE EMBRYONIC STEM CELLS AND MODULATES DIFFERENTIATION OUTCOMES IN A CONTEXT-DEPENDENT MANNER

The unique cell cycle properties of pluripotent stem cells have been linked to their identity and postulated to be essential for pluripotency maintenance. ZD7288, an inhibitor of the HCN channel family, has previously been reported to modulate cell cycle progression in mouse embryonic stem cells (ESCs), however the effects of this compound and the cell cycle perturbation it caused on cell fate were not described. This paper investigates the cell cycle dynamics and cell fate of mouse ESCs treated with ZD7288.

4.2.1 ZD7288, an HCN channel blocker, reduced ESC proliferation by increasing doubling time due to extended G1 and S phases

ESCs cultured in maintenance media with the addition of ZD7288 were found to be significantly fewer after 4 days of culture, with a reduction in cell number of over 50% as compared to control culture conditions, accompanied by only a disproportionally small reduction in viability, in line with the previously described action of the compound[150]. Similarly, cells plated at subclonal densities and cultured in the presence of the compound formed significantly smaller colonies (27 vs 44 cells per colony in treated and control cells,

respectively). Reduced cell numbers could be explained by fewer proliferating cells or longer cell cycle. G0 is usually absent from ESCs, but to ensure that all cells were in active cell cycle, cells were stained for KI-67, a marker of active proliferation. As expected, no differences were observed, suggesting that the cell cycle was longer in ZD7288 treated cells. Based on these cell number observations, doubling time for treated cultures was calculated to be ≈37 hours, as compared to ≈28 hours for control cells, in line with previous reports[99].

To examine what accounted for the increased cell cycle length, we performed DNA content analysis using flow cytometry. This showed a significant increase in the fraction of cell in S-phase (37.6 % in control, 41.8 % in treated) and a corresponding decrease in G2/M phase (28.3 % in control, 23.4 % in treated) in the treated cultures. Taken together with the calculated doubling time, this corresponds to an increase in the length of G1 and S phases in response to ZD7288 treatment. This was in contrast to what had previously been reported. Previous report by Lau *et al*[150] indicated an accumulation in S-phase with a compensatory decrease in the G1 fraction, indicating an increase in the length of S-phase only. Interestingly, the cell cycle length in the previous publication was much shorter than in the present one, possibly due to difference in culture conditions. Lau *et al*[150] used serum-based, whereas this study used serum-free media, and it has previously been reported that ESC doubling time varied between 13 and 34 hours depending on media formulation, with serum-free medias resulting in longer doubling times.

4.2.2 DNA replication is altered in ZD7288 treated cells

Surprisingly, the number of 5-ethynyl-2´-deoxyuridine (EdU) incorporating cells, a marker of cells in S-phase, appeared to be lower in treated cultures when examined using flow cytometry. This was puzzling because the number of S-phase cells was increased in treated cultures based on DNA content quantification, prompting us to take a closer look at the at EdU incorporation pattern. EdU staining pattern was distinctly different in the treated cells, which formed a single wide peak, instead of the two peaks corresponding to positive and negative cells usually seen, and observed in the control.

Potential causes of this alteration could be intra-S phase stalling, reduced DNA replication, or reduced availability of the EdU nucleotide. To investigate the possibility of intra-S-phase stalling, cells were staged as “early” or “late” S-phase based on EdU incorporation pattern following visualization using confocal microscopy. The fraction of “early” phase cells was similar in both conditions (55.7 % in control and 53.3 % in treated culture), suggesting altered EdU availability or slower DNA replication, which would be in line with the observed

prolonged S-phase transit time. It is possible that the two proposed scenarios are linked, with reduced nucleoside availability hampering replication. Alternatively, ion concentrations are known to affect DNA tertiary and quaternary structure, altered sodium or potassium concentrations due to HCN inhibition could induce structural changes attenuating replication speed. Finally, DNA replication speed varies during S-phase[196], and the observed effect may be related to modulation of a mechanism involved in DNA replication speed regulation.

4.2.3 Treatment with ZD7288 decreases colony formation frequency but does not alter expression of the core pluripotency genes and effects cell differentiation in a context dependent manner

To test the effects of cell cycle perturbation on pluripotency, cells were plated at subclonal density to test clonogenicity and self-renewal. The number of colonies formed per well was much lower in the treated condition, and more colonies had rough edges, which could be a sign of differentiation. To test this, cells were stained for the various markers of pluripotency (SSEA1, OCT3/4, SOX2, NANOG, and ALP activity), with no significant changes detected. Similarly, no changes in pluripotency marker expression were detected on an RNA level. To functionally test maintenance of pluripotency in ZD7288 treated cultures, treated cells were re-plated and both colony formation rates and colony morphology were restored to control levels. A possible explanation for the observed effect is altered attachment/cytoskeletal dynamics, which could affect both the propensity of the cells to attach and form colonies and cellular morphology on colony borders.

To functionally test the effects of ZD7288 on the other cardinal stem cell property – differentiation, ESCs were induced to differentiate either spontaneously in fetal bovine serum (FBS) –containing media with LIF withdrawal, or toward the neural lineage as per defined serum-free protocol. Spontaneous differentiation was potentiated by ZD7288 addition, with more pronounced downregulation of the pluripotency markers and upregulation of most lineage markers. Surprisingly, neural differentiation did not benefit from ZD7288 addition, with similar levels of expression of most pluripotency factors in control and treated cells, and a less efficient induction of 2 of the 3 ectoderm markers tested. These findings suggest that the action of ZD7288 in differentiation is context dependent. This is rather similar to the difference in cell cycle effects between the previous and current reports. Intriguingly, one of the most striking differences is also similar – serum-containing media. Although the underlying mechanisms of these differences are unclear, this work adds to the weight of

evidence that extending G1 does not necessarily result in differentiation, at least not if the G1-S ratio is maintained[197].

In summary, we found that ZD7288, an inhibitor of the HCN channel family, reduced ESC proliferation by extending G1 and S residence time. This did not compromise cell identity, with treated cell maintaining pluripotency and the ability to differentiate to all lineages. Differentiation in FBS-containing media was facilitated by the compound, while directed differentiation to neuroectoderm in defined media was less efficient in its presence.

4.3 PAPER III: HYPERPOLARIZATION-ACTIVATED CYCLIC NUCLEOTIDE-GATED CHANNELS MODULATE ACTIVE PROLIFERATION AND METABOLISM, AND MAINTAIN MOLECULAR CLOCK OSCILLATIONS IN ADULT MOUSE NEURAL STEM PROGENITOR CELLS

Data from recent sequencing studies attempting to define genes specific to the activated and quiescent stem cell states have indicated that a number of ion channels are differentially expressed in the two pools, with members of the HCN family on the list. Taken together with the established fact that voltage gated potassium channels are involved in cell cycle regulation, and data from other stem cells systems, including Paper II above, this work set out to investigate a potential role for HCN family channels in adult mouse NSPC proliferation.

4.3.1 HCN2 and 3 are expressed throughout the NSPC hierarchy with their function restricted to the proliferative (S and G2/M) phases

Gene expression analysis with qRT-PCR indicated that *Hcn2* and *Hcn3* were expressed in both bulk primary SVZ NSPCs, and in all populations of NSPCs sorted according to GFAP::GFP intensity, believed to correspond to different levels in the NSPC differentiation hierarchy, including type B and C cells. This was promising, as HCN3 was the isoform previously reported to modulate ESC proliferation, confirmed in **paper II**, and was identified as part of the quiescent stem cell signature in data from Codega *et al*[198]. While expression of HCN3 was not confirmed on a protein level due to technical limitations, HCN2 co-stained BrdU+/SOX2+ NSPCs in tissue sections from the SVZ and SGZ. To further verify channel expression, function was assessed with electrophysiology. The typical hyperpolarization-activated current (I_h), sensitive to the specific blocker ZD7288, could be recorded from some, but not all NSPCs.

To investigate the basis for non-uniform presence of I_h, cells were cell-cycle sorted based on DNA content. This showed that while no cells in the G0/G1 cell cycle fraction carried the

current, the current was present in many S, and the majority of G2/M cells. Correspondingly, HCN2 staining intensity was found to be higher in those cell cycle phases as ascertained by flow cytometry, and differential channel localization was observed in the three cell cycle phases by confocal microscopy. The association of HCN function with the proliferative phases further supported the hypothesis that the channel could be implicated in the regulation of proliferation.

4.3.2 Inhibition of HCN channel activity attenuated NSPC proliferation due to a reversible G0/G1 block *in vitro* and its effects extend *in vivo*

Culture of primary SVZ NSPCs in the presence of ZD7288, an HCN channel inhibitor, resulted in smaller neurosphere size, while block of HCN function with different concentrations of its inhibitors ZD7288, Zatebradine, or CsCl resulted in lower SVZ NSPC number as determined by a viability assay. To ensure the difference in cell numbers was not due to cell death, as HCN has been reported to regulate apoptosis[189], levels of activated caspase-3 and the subG1 fraction of cell cycle were quantified. No significant induction of cell death was observed. Moreover, EdU incorporation was significantly reduced (by 46%) in treated cells 24 hours after treatment initiation. The proliferative effects were confirmed on a genetic level with si- and shRNA. Rather conflictingly, siRNA showed that HCN2 but not HCN3 knockdown reduced cell numbers, while shRNA, showed that knockdown of either gene was sufficient to see a reduction in proliferating cells. This could be due to different potency of knockdown, but regardless, confirmed the expectation that HCN activity is involved in regulation of NSPC proliferation.

To further investigate the effect of HCN inhibition on the cell cycle, DNA content analysis of treated and untreated cells was performed, indicating an increase in G0/G1 cell cycle fraction accompanied by a reduction in the S and G2/M. To further identify whether it was G0 or G1 that was affected, cells were stained for KI-67, which showed an increase in cells no longer in active proliferation, as indicated by loss of KI-67 marker expression. This was further supported by increased survival of ZD7288 treated NSPCs upon long term (48 hours) exposure to the genotoxic agent arabinofuranosyl cytidine (Ara-C), and the full reversibility of the induced proliferative block upon washout. The reversibility of the proliferative effect distinguished the observed block as quiescence, as opposed to senescence.

Importantly, similar effects on proliferation (reduction in proliferation by BrdU incorporation, phospho-histone 3 (a marker of M-phase) expression, and KI-67 expression) were seen *in vivo* in both SVZ and SGZ when animals were injected with 4mg/kg of ZD7288.

This was not accompanied by induction of apoptosis, with onset of action observed at 24 hours and complete attenuation of the effect at 72 hours, further supporting reversibility.

4.3.3 Transcriptome analysis identified alterations in expression of cell cycle regulators, metabolic enzymes, and members of the molecular clock in treated cells, while NSPC identify marker expression was not altered

Transcriptome analysis confirmed that treated cells were not undergoing differentiation, as expression of key NSPC regulatory genes *Sox2*, *Pax6* and *Msi1* was similar in treated and untreated cells, and expression of differentiation genes was not induced by treatment. Markers of quiescence and activation, on the other hand, were altered. *Nestin* and *Ascl1*, both implicated in activation and stem cell proliferation, were downregulated on an RNA level, while western blot for EGFR indicated that the receptor was downregulated on a protein level. Inhibition of proliferation was further confirmed by reduced expression of many positive cell cycle regulators in treated cells, while some negative regulators of proliferation were upregulated. p21 and p27 canonical inducers of cell cycle arrest, were upregulated on an RNA, but not protein level, suggesting a novel mechanism for induction of quiescence in response to HCN inhibition.

Gene Ontology (GO) term enrichment analysis of differentially expressed genes identified metabolism as one of the major gene categories altered in treated cells. Enzymes involved in glycolysis were strongly downregulated. To complement this analysis and look at metabolism more functionally, we analyzed metabolite abundance. Levels of glycolysis intermediates, lactate, and amino acids were found to be significantly altered, confirming a metabolic perturbation as either the mediator or a consequence of the effects of HCN inhibition.

When a more narrow approach was taken GO enrichment analysis was run on genes shared between the quiescence signature published by Codega *et al*[198] and those altered in response to ZD7288. "Rhythmic processes", containing many genes known to be components of the molecular clock network (*Per1*, *Per2*, *Cry1*, *Cry2*, *Nr1d1* and *Nr1d2* (Rev-Erb-α and β), *Timeless* and *Tipin*), was the most significantly enriched term. To test functional significance of the observed change, luminescence from Per2-driven luciferase was recorded in adherent NSPCs and SVZ explants with and without ZD7288 in the media. In line with observations of deregulation on the transcriptional level, clock oscillations were abolished upon HCN inhibition. In light of the previous reports of clock function regulating proliferation in other stem cell systems[199,200], we tested whether ZD7288 effect could be phenocopied by activation

of Rev-Erb- α, the most upregulated component of the clock network identified in our dataset. Treatment of NSPCs with the Rev-Erb- α agonist SR9009 resulted in reduced proliferation, suggesting its involvement in mediating the effect of HCN inhibition. Interestingly, hippocampus hyperproliferation was reported in Rev-Erb- α knockout animals[201], supporting its function as a regulator of proliferation in NSPCs. It has also been reported to be inhibited by lithium[202], representing an alternative pathway for proliferative increase seen in **paper I**. Although it is not clear if or how the observed changes in metabolism and the molecular clock are related, recent publications have reported metabolic perturbations in the kidneys of clock-null animals[203].

4.3.4 Pre-treatment of juvenile mice with ZD7288 and a neuroprotective effect, reducing the deleterious effects of ionizing radiation.

Following up on work in **paper I**, neuroprotective potential of HCN blockade was tested in an animal model of ionizing radiation (IR)-induced NSPC damage. Juvenile animals (post-natal day 8) were treated with ZD7288 (8 mg/kg) 24 hours prior to exposure to 2Gy of IR. Animals were sacrificed 6 hours following exposure and levels of proliferation and cell death were examined in the neurogenic areas (SGZ and SVZ). The levels of apoptosis as seen by activated caspase-3 staining were significantly reduced, and proliferation as measured by BrdU incorporation was similar in treated and untreated animals. No cell cycle analysis was performed, as in **paper I**, to assess potential additional stalling of cells in G0/G1, but similar proliferation rates in both irradiated and non-irradiated animals suggest that proliferation was not affected by IR, possibly due to the fact that the most sensitive cells were in proliferative arrest due to ZD7288 treatment, and thus protected from IR damage and the apoptosis that would have followed. An alternative explanation of HCN inhibitor action could be related to calcium influx-induced apoptosis, in which HCN2 was previously implicated.

To test whether there may be a therapeutic window with HCN inhibition, and a similar protective effect would not be observed for cancer cells, 17 patient-derived cancer stem cell lines were tested for growth response following culture in media containing 0-49 μM ZD2788. Across all lines there was no change in viability signal to any of the tested inhibitor concentrations, suggesting that HCN has no proliferative effect in cancer stem cells. As the cell line tested were of human origin, while all other work had been done on murine cells, to ensure the observed lack of effect was not due to species differences, proliferation kinetics in response to ZD7288 treatment were tested in human fetal neural stem cells (hfNSCs) and human glioma-initiating cancer stem cells (giCSCs). Confirming the viability test results, the

number of EdU positive giCSCs did not change in response to ZD7288, while proliferation of hfNSCs was inhibited resulting in fewer EdU incorporating cells, as observed earlier in mouse NSPCs. The differential response of cancer and tissue stem cells to HCN inhibition could reflect abnormal cell cycle regulation in cancer cells[204], but also disrupted clock function, which has been reported previously[205]. Finally, alterations in metabolism, a hallmark of cancer[206], is also a possible explanation of observed differences.

In summary, HCN2 and HCN3 are expressed in mouse NSPCs with function restricted to the S and G2/M phases. Their inhibition inhibited proliferation inducing a reversible G0/G1 accumulation accompanied by changes in markers of quiescence/activation and cell cycle, but not stem cell identity. This was accompanied by downregulation of glycolysis and changes in metabolite levels, as well as abolition of molecular clock oscillations due to upregulation of negative clock components. An agonist of the peripheral clock component Rev-erb-α could mimic the proliferative effects of HCN inhibition. This effect could be confirmed in both proliferative regions *in vivo*, and resulted in reduced cell death in juvenile animals pre-treated with ZD7288 prior to IR exposure.

5 CONCLUSION AND FUTURE DIRECTIONS

The work in this thesis showed that:

I. Lithium induces proliferation of juvenile mouse NSPCs *in vitro*, and although it does not reduce apoptosis, it protects them from IR-induced DNA damage, and induces their return to proliferation following G1 block

II. Treatment of mouse embryonic stem cells with the HCN blocker ZD7288 attenuates their proliferation by extending their cell cycle, specifically in the G1 and S phases. Treatment does not compromise pluripotency under maintenance conditions, but can facilitate differentiation in a context-dependent manner.

III. HCN2 and HCN3 are expressed in NSPCs and functional in the S and G2/M phases. Block of their activity reduces proliferation *in vivo* and *in vitro* by inducing a reversible cell cycle block in the G0/G1 phase, which exhibits many features of quiescence: reducing glycolytic metabolism, downregulating markers of activation, and maintenance of NSPC identity. I_h current block also disrupts oscillations of the molecular clock, with modulation of the clock component Rev-erb-α having similar effects on proliferation.

IV. HCN activity can protect NSPCs from genotoxic stress both *in vitro* and *in vivo*, and attenuates IR-induced apoptosis *in vivo*. HCN inhibition may have the potential to be developed into a neuroprotective therapy for pediatric cancer patients.

Additional work exploring the mechanisms behind these finding and investigating their potential translation to the clinic is needed. The work presented here makes a case for modulation of ion-dependent processes as an in-point for cell state regulation. Modulation of ion availability as a co-factor (in the case of lithium effects) or ion trafficking (in the case of HCN inhibition) have both been shown to be viable strategies for regulation, although the downstream mechanisms and exact targets of each intervention remain to be elucidated. An interesting area of overlap is metabolism, which is regulated in a circadian manner both on a whole-organism and on the cell level[207], and is known to regulate stem cell dynamics, both in adult stem cell systems[208,209] and in the embryo through diapause and reprogramming[210,211]. It is however unclear how the metabolic and circadian regulatory systems relate to each other and proliferation – are these parallel processes that can exert proliferative control? Is one process subservient to the other? Are perturbations in one system just a side effect of the

deregulation of the other? The effect observed in ESCs in **paper II**, if mediated in a similar fashion to the NSPC effect in **paper III**, would put metabolism at the top of the hierarchy, and circadian perturbations as a side effect, because ESCs lack a circadian clock[212]. It is unclear how well this would generalize to somatic cells, as ESCs also lack conventional cell cycle control. To more appropriately address this question, it would be interesting to test how the effects of HCN inhibition observed in **paper III** would change in BMAL1-deficient mice, which lack circadian rhythmicity[213], but not the usual somatic cell cycle machinery. To more closely examine the interaction of HCN-inhibition, metabolism, and proliferation, it would be interesting to follow up the work in **paper II** on proliferative slow down of ESCs with further experiments looking at metabolic effects in that cell system. ESCs are a relatively homogenous and robust cell system, while still relevant in a developmental context, making them a good platform for research into fundamentals of cellular physiology.

This thesis also investigated two very different approaches to neuroprotection – temporary attenuation of proliferation (**paper III**) and a combination of DNA damage protection and induction of proliferation to overcome cell loss due to IR treatment (**paper I**). In addition to developing compounds and techniques for more precise delivery of active compounds to injury site, this work could be followed-up by investigations into how these two interventions may be combined to improve outcomes. Pre-treatment with an HCN inhibitor in combination with acute or follow-up treatment with lithium may be a worthwhile approach to neuroprotection, taking advantage of both strategies to overcome IR-induced damage. Since significant additional work would be needed to characterize such a regiment, lithium is likely a more promising first approach for clinical tests of neuroprotection due to its fairly well understood toxicity profile. However, long-term implications of lithium use should first be more thoroughly investigated. Deregulation of proliferation can lead to tumorigenicity, especially in combination with genotoxicity and stress, which NSPCs face following IR treatment. Ideally, development of therapy should be guided by mechanistic insights into the mode of action of both lithium and HCN inhibition, until then, the empirical approach should not be overlooked.

6 ACKNOWLEDGEMENTS

I would like to thank my main supervisor Michael Andäng, and co-supervisors Helena Johard, Gabriella Lundkvist, and Per Uhlen for giving me the opportunity to undertake a Ph.D. at the Karolinska Institute under their guidance.

I would like to extend a special thanks my collaborators, in particular Petra Sekyrova, Giulia Zanni, and Fei Gao, whose hard work, insightful input, and dedication led to the publications and manuscript in this thesis.

I would also like to thank all members of the Andäng group and the Division of Molecular Neurobiology for the supportive environment and fun times.

Lastly, although for the most part not directly involved in this work, it is really my friends and family who made it possible. I take this opportunity to thank you in writing and let you know that I am always grateful for your help, support, and for the privilege of knowing you. You've all left a unique imprint on who I am and will no doubt continue to do so not matter how short or long the distance that separates us. To new beginnings and enduring bonds.

7 REFERENCES

1. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414(6859):105-111.
2. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature.* 1963;197:452-454.
3. Rezza A, Sennett R, Rendl M. Adult stem cell niches: cellular and molecular components. *Current topics in developmental biology.* 2014;107:333-372.
4. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell.* 2008;132(4):631-644.
5. Maslova MN, Tavrovskaia TV. [The seasonal dynamics of erythropoiesis in the frog Rana temporaria]. *Zhurnal evoliutsionnoi biokhimii i fiziologii.* 1993;29(2):211-214.
6. Gage FH, Temple S. Neural stem cells: generating and regenerating the brain. *Neuron.* 2013;80(3):588-601.
7. Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *The Journal of comparative neurology.* 1965;124(3):319-335.
8. Altman J. Autoradiographic investigation of cell proliferation in the brains of rats and cats. *The Anatomical record.* 1963;145:573-591.
9. Altman J, Das GD. Postnatal neurogenesis in the guinea-pig. *Nature.* 1967;214(5093):1098-1101.
10. Eriksson PS, Perfilieva E, Bjork-Eriksson T, et al. Neurogenesis in the adult human hippocampus. *Nature medicine.* 1998;4(11):1313-1317.
11. Gould E. How widespread is adult neurogenesis in mammals? *Nature reviews. Neuroscience.* 2007;8(6):481-488.
12. Jellinger KA. Adult Neurogenesis: Stem cells and neuronal development in the adult brain. *European Journal of Neurology.* 2007;14(3):e13-e13.
13. Ming GL, Song H. Adult neurogenesis in the mammalian central nervous system. *Annual review of neuroscience.* 2005;28:223-250.
14. Consiglio A, Gritti A, Dolcetta D, et al. Robust in vivo gene transfer into adult mammalian neural stem cells by lentiviral vectors. *Proceedings of the National Academy of Sciences of the United States of America.* 2004;101(41):14835-14840.
15. Shook BA, Manz DH, Peters JJ, Kang S, Conover JC. Spatiotemporal changes to the subventricular zone stem cell pool through aging. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2012;32(20):6947-6956.
16. Hamilton LK, Joppe SE, L MC, Fernandes KJ. Aging and neurogenesis in the adult forebrain: what we have learned and where we should go from here. *The European journal of neuroscience.* 2013;37(12):1978-1986.
17. Spalding KL, Bergmann O, Alkass K, et al. Dynamics of hippocampal neurogenesis in adult humans. *Cell.* 2013;153(6):1219-1227.
18. Ge S, Sailor KA, Ming GL, Song H. Synaptic integration and plasticity of new neurons in the adult hippocampus. *The Journal of physiology.* 2008;586(16):3759-3765.

19. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron.* 2011;70(4):687-702.
20. Sahay A, Wilson DA, Hen R. Pattern separation: a common function for new neurons in hippocampus and olfactory bulb. *Neuron.* 2011;70(4):582-588.
21. Lledo PM, Alonso M, Grubb MS. Adult neurogenesis and functional plasticity in neuronal circuits. *Nature reviews. Neuroscience.* 2006;7(3):179-193.
22. Akers KG, Martinez-Canabal A, Restivo L, et al. Hippocampal neurogenesis regulates forgetting during adulthood and infancy. *Science (New York, N.Y.).* 2014;344(6184):598-602.
23. Jiang W, Zhang Y, Xiao L, et al. Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *The Journal of clinical investigation.* 2005;115(11):3104-3116.
24. Santarelli L, Saxe M, Gross C, et al. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science (New York, N.Y.).* 2003;301(5634):805-809.
25. Jessberger S, Zhao C, Toni N, Clemenson GD, Jr., Li Y, Gage FH. Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2007;27(35):9400-9407.
26. Lindvall O, Kokaia Z. Neurogenesis following Stroke Affecting the Adult Brain. *Cold Spring Harbor perspectives in biology.* 2015;7(11).
27. Lindvall O, Bjorklund A. Cell therapy in Parkinson's disease. *NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics.* 2004;1(4):382-393.
28. Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS. Adrenal hormones suppress cell division in the adult rat dentate gyrus. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 1992;12(9):3642-3650.
29. Perera TD, Coplan JD, Lisanby SH, et al. Antidepressant-induced neurogenesis in the hippocampus of adult nonhuman primates. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2007;27(18):4894-4901.
30. Anderson NE. Late complications in childhood central nervous system tumour survivors. *Current opinion in neurology.* 2003;16(6):677-683.
31. Armstrong GT, Liu Q, Yasui Y, et al. Long-term outcomes among adult survivors of childhood central nervous system malignancies in the Childhood Cancer Survivor Study. *J Natl Cancer Inst.* 2009;101(13):946-958.
32. Monje ML, Palmer T. Radiation injury and neurogenesis. *Current opinion in neurology.* 2003;16(2):129-134.
33. Ji JF, Ji SJ, Sun R, et al. Forced running exercise attenuates hippocampal neurogenesis impairment and the neurocognitive deficits induced by whole-brain irradiation via the BDNF-mediated pathway. *Biochemical and biophysical research communications.* 2014;443(2):646-651.
34. Oh SB, Park HR, Jang YJ, Choi SY, Son TG, Lee J. Baicalein attenuates impaired hippocampal neurogenesis and the neurocognitive deficits induced by gamma-ray radiation. *British journal of pharmacology.* 2013;168(2):421-431.
35. Huo K, Sun Y, Li H, et al. Lithium reduced neural progenitor apoptosis in the hippocampus and ameliorated functional deficits after irradiation to the immature mouse brain. *Molecular and cellular neurosciences.* 2012;51(1-2):32-42.

36. Paridaen JT, Huttner WB. Neurogenesis during development of the vertebrate central nervous system. *EMBO reports.* 2014;15(4):351-364.
37. Gao P, Postiglione MP, Krieger TG, et al. Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell.* 2014;159(4):775-788.
38. Hansen DV, Lui JH, Parker PR, Kriegstein AR. Neurogenic radial glia in the outer subventricular zone of human neocortex. *Nature.* 2010;464(7288):554-561.
39. Li G, Fang L, Fernandez G, Pleasure SJ. The ventral hippocampus is the embryonic origin for adult neural stem cells in the dentate gyrus. *Neuron.* 2013;78(4):658-672.
40. Fuentealba LC, Rompani SB, Parraguez JI, et al. Embryonic Origin of Postnatal Neural Stem Cells. *Cell.* 2015;161(7):1644-1655.
41. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell.* 1999;97(6):703-716.
42. Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH. In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell stem cell.* 2007;1(5):515-528.
43. Zhao C, Deng W, Gage FH. Mechanisms and functional implications of adult neurogenesis. *Cell.* 2008;132(4):645-660.
44. Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science (New York, N.Y.).* 1992;255(5052):1707-1710.
45. Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 1999;19(19):8487-8497.
46. Malatesta P, Hartfuss E, Gotz M. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development (Cambridge, England).* 2000;127(24):5253-5263.
47. Ortega F, Gascon S, Masserdotti G, et al. Oligodendrogliogenic and neurogenic adult subependymal zone neural stem cells constitute distinct lineages and exhibit differential responsiveness to Wnt signalling. *Nature cell biology.* 2013;15(6):602-613.
48. Calzolari F, Michel J, Baumgart EV, Theis F, Gotz M, Ninkovic J. Fast clonal expansion and limited neural stem cell self-renewal in the adult subependymal zone. *Nature neuroscience.* 2015;18(4):490-492.
49. Encinas JM, Michurina TV, Peunova N, et al. Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell stem cell.* 2011;8(5):566-579.
50. Bonaguidi MA, Wheeler MA, Shapiro JS, et al. In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell.* 2011;145(7):1142-1155.
51. Suhonen JO, Peterson DA, Ray J, Gage FH. Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. *Nature.* 1996;383(6601):624-627.

52. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell proliferation.* 2003;36(3):131-149.
53. Murray AW. Recycling the cell cycle: cyclins revisited. *Cell.* 2004;116(2):221-234.
54. Barnum KJ, O'Connell MJ. Cell cycle regulation by checkpoints. *Methods in molecular biology (Clifton, N.J.).* 2014;1170:29-40.
55. Shin J, Ming GL, Song H. Decoding neural transcriptomes and epigenomes via high-throughput sequencing. *Nature neuroscience.* 2014;17(11):1463-1475.
56. Jang MH, Bonaguidi MA, Kitabatake Y, et al. Secreted frizzled-related protein 3 regulates activity-dependent adult hippocampal neurogenesis. *Cell stem cell.* 2013;12(2):215-223.
57. Song J, Zhong C, Bonaguidi MA, et al. Neuronal circuitry mechanism regulating adult quiescent neural stem-cell fate decision. *Nature.* 2012;489(7414):150-154.
58. Daynac M, Chicheportiche A, Pineda JR, Gauthier LR, Boussin FD, Mouthon MA. Quiescent neural stem cells exit dormancy upon alteration of GABAAR signaling following radiation damage. *Stem cell research.* 2013;11(1):516-528.
59. Walker TL, Wierick A, Sykes AM, et al. Prominin-1 allows prospective isolation of neural stem cells from the adult murine hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2013;33(7):3010-3024.
60. Pastrana E, Cheng LC, Doetsch F. Simultaneous prospective purification of adult subventricular zone neural stem cells and their progeny. *Proceedings of the National Academy of Sciences of the United States of America.* 2009;106(15):6387-6392.
61. Candelario KM, Shuttleworth CW, Cunningham LA. Neural stem/progenitor cells display a low requirement for oxidative metabolism independent of hypoxia inducible factor-1alpha expression. *Journal of neurochemistry.* 2013;125(3):420-429.
62. Diaz-Castro B, Pardal R, Garcia-Flores P, et al. Resistance of glia-like central and peripheral neural stem cells to genetically induced mitochondrial dysfunction--differential effects on neurogenesis. *EMBO reports.* 2015;16(11):1511-1519.
63. Llorens-Bobadilla E, Zhao S, Baser A, Saiz-Castro G, Zwadlo K, Martin-Villalba A. Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury. *Cell stem cell.* 2015;17(3):329-340.
64. Hsieh J. Orchestrating transcriptional control of adult neurogenesis. *Genes & development.* 2012;26(10):1010-1021.
65. Mateo JL, van den Berg DL, Haeussler M, et al. Characterization of the neural stem cell gene regulatory network identifies OLIG2 as a multifunctional regulator of self-renewal. *Genome research.* 2015;25(1):41-56.
66. Andersen J, Urban N, Achimastou A, et al. A transcriptional mechanism integrating inputs from extracellular signals to activate hippocampal stem cells. *Neuron.* 2014;83(5):1085-1097.
67. Liu HK, Belz T, Bock D, et al. The nuclear receptor tailless is required for neurogenesis in the adult subventricular zone. *Genes & development.* 2008;22(18):2473-2478.

68. Borgs L, Beukelaers P, Vandenbosch R, et al. Period 2 regulates neural stem/progenitor cell proliferation in the adult hippocampus. *BMC neuroscience.* 2009;10:30.
69. Martynoga B, Mateo JL, Zhou B, et al. Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence. *Genes & development.* 2013;27(16):1769-1786.
70. Wu H, Coskun V, Tao J, et al. Dnmt3a-dependent nonpromoter DNA methylation facilitates transcription of neurogenic genes. *Science (New York, N.Y.).* 2010;329(5990):444-448.
71. Jones KM, Saric N, Russell JP, Andoniadou CL, Scambler PJ, Basson MA. CHD7 maintains neural stem cell quiescence and prevents premature stem cell depletion in the adult hippocampus. *Stem cells (Dayton, Ohio).* 2015;33(1):196-210.
72. Andreu Z, Khan MA, Gonzalez-Gomez P, et al. The cyclin-dependent kinase inhibitor p27 kip1 regulates radial stem cell quiescence and neurogenesis in the adult hippocampus. *Stem cells (Dayton, Ohio).* 2015;33(1):219-229.
73. Sun Y, Hu J, Zhou L, Pollard SM, Smith A. Interplay between FGF2 and BMP controls the self-renewal, dormancy and differentiation of rat neural stem cells. *Journal of cell science.* 2011;124(Pt 11):1867-1877.
74. Ottone C, Krusche B, Whitby A, et al. Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells. *Nature cell biology.* 2014;16(11):1045-1056.
75. Posfai E, Tam OH, Rossant J. Mechanisms of pluripotency in vivo and in vitro. *Current topics in developmental biology.* 2014;107:1-37.
76. Fenelon JC, Banerjee A, Murphy BD. Embryonic diapause: development on hold. *The International journal of developmental biology.* 2014;58(2-4):163-174.
77. Fu Z, Wang B, Wang S, et al. Integral proteomic analysis of blastocysts reveals key molecular machinery governing embryonic diapause and reactivation for implantation in mice. *Biology of reproduction.* 2014;90(3):52.
78. Lee JE, Oh HA, Song H, et al. Autophagy regulates embryonic survival during delayed implantation. *Endocrinology.* 2011;152(5):2067-2075.
79. Liu WM, Pang RT, Cheong AW, et al. Involvement of microRNA lethal-7a in the regulation of embryo implantation in mice. *PloS one.* 2012;7(5):e37039.
80. Lopes FL, Desmarais JA, Murphy BD. Embryonic diapause and its regulation. *Reproduction (Cambridge, England).* 2004;128(6):669-678.
81. Boroviak T, Loos R, Lombard P, et al. Lineage-Specific Profiling Delineates the Emergence and Progression of Naive Pluripotency in Mammalian Embryogenesis. *Developmental cell.* 2015;35(3):366-382.
82. Ying QL, Wray J, Nichols J, et al. The ground state of embryonic stem cell self-renewal. *Nature.* 2008;453(7194):519-523.
83. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126(4):663-676.
84. Nichols J, Smith A. Pluripotency in the embryo and in culture. *Cold Spring Harbor perspectives in biology.* 2012;4(8):a008128.
85. Chambers I, Tomlinson SR. The transcriptional foundation of pluripotency. *Development (Cambridge, England).* 2009;136(14):2311-2322.

86. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell.* 2007;131(5):861-872.
87. de Rham C, Villard J. Potential and limitation of HLA-based banking of human pluripotent stem cells for cell therapy. *Journal of immunology research.* 2014;2014:518135.
88. Williams RL, Hilton DJ, Pease S, et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature.* 1988;336(6200):684-687.
89. Ying QL, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell.* 2003;115(3):281-292.
90. Krawchuk D, Honma-Yamanaka N, Anani S, Yamanaka Y. FGF4 is a limiting factor controlling the proportions of primitive endoderm and epiblast in the ICM of the mouse blastocyst. *Developmental biology.* 2013;384(1):65-71.
91. Kunath T, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development (Cambridge, England).* 2007;134(16):2895-2902.
92. Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3alpha and GSK-3beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Developmental cell.* 2007;12(6):957-971.
93. Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell.* 2008;132(4):661-680.
94. Kamao H, Mandai M, Okamoto S, et al. Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. *Stem cell reports.* 2014;2(2):205-218.
95. Kimbrel EA, Lanza R. Current status of pluripotent stem cells: moving the first therapies to the clinic. *Nature reviews. Drug discovery.* 2015;14(10):681-692.
96. Fan Y, Wu J, Ashok P, Hsiung M, Tzanakakis ES. Production of human pluripotent stem cell therapeutics under defined xeno-free conditions: progress and challenges. *Stem cell reviews.* 2015;11(1):96-109.
97. Snow MHL. Gastrulation in the mouse: Growth and regionalization of the epiblast. *Development (Cambridge, England).* 1977;42(1):293-303.
98. Stead E, White J, Faast R, et al. Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. *Oncogene.* 2002;21(54):8320-8333.
99. Tamm C, Pijuan Galito S, Anneren C. A comparative study of protocols for mouse embryonic stem cell culturing. *PloS one.* 2013;8(12):e81156.
100. Hernandez L, Kozlov S, Piras G, Stewart CL. Paternal and maternal genomes confer opposite effects on proliferation, cell-cycle length, senescence, and tumor formation. *Proceedings of the National Academy of Sciences of the United States of America.* 2003;100(23):13344-13349.
101. Yang VS, Carter SA, Hyland SJ, Tachibana-Konwalski K, Laskey RA, Gonzalez MA. Geminin escapes degradation in G1 of mouse pluripotent cells and mediates the expression of Oct4, Sox2, and Nanog. *Current biology : CB.* 2011;21(8):692-699.

102. Ballabeni A, Park IH, Zhao R, et al. Cell cycle adaptations of embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(48):19252-19257.
103. Savatier P, Huang S, Szekely L, Wiman KG, Samarut J. Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. *Oncogene.* 1994;9(3):809-818.
104. Schratt G, Weinhold B, Lundberg AS, et al. Serum response factor is required for immediate-early gene activation yet is dispensable for proliferation of embryonic stem cells. *Molecular and cellular biology.* 2001;21(8):2933-2943.
105. Faast R, White J, Cartwright P, Crocker L, Sarcevic B, Dalton S. Cdk6-cyclin D3 activity in murine ES cells is resistant to inhibition by p16(INK4a). *Oncogene.* 2004;23(2):491-502.
106. Lin T, Chao C, Saito S, et al. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nature cell biology.* 2005;7(2):165-171.
107. Mantel C, Guo Y, Lee MR, et al. Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotpic instability. *Blood.* 2007;109(10):4518-4527.
108. Neganova I, Tilgner K, Buskin A, et al. CDK1 plays an important role in the maintenance of pluripotency and genomic stability in human pluripotent stem cells. *Cell death & disease.* 2014;5:e1508.
109. Coronado D, Godet M, Bourillot PY, et al. A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency. *Stem cell research.* 2013;10(1):118-131.
110. Guo S, Zi X, Schulz VP, et al. Nonstochastic reprogramming from a privileged somatic cell state. *Cell.* 2014;156(4):649-662.
111. Ruiz S, Panopoulos AD, Herrerias A, et al. A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. *Current biology : CB.* 2011;21(1):45-52.
112. Singh AM, Chappell J, Trost R, et al. Cell-cycle control of developmentally regulated transcription factors accounts for heterogeneity in human pluripotent cells. *Stem cell reports.* 2013;1(6):532-544.
113. Li H, Collado M, Villasante A, et al. p27(Kip1) directly represses Sox2 during embryonic stem cell differentiation. *Cell stem cell.* 2012;11(6):845-852.
114. Li VC, Ballabeni A, Kirschner MW. Gap 1 phase length and mouse embryonic stem cell self-renewal. *Proceedings of the National Academy of Sciences of the United States of America.* 2012;109(31):12550-12555.
115. Li VC, Kirschner MW. Molecular ties between the cell cycle and differentiation in embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2014;111(26):9503-9508.
116. Eaton DC. Ionic channels of excitable membranes. Bertil Hille. Sunderland, Ma: Sinauer Associates, 1984. *Journal of Neuroscience Research.* 1985;13(4):599-600.
117. Deng D, Yan N. GLUT, SGLT, and SWEET: Structural and mechanistic investigations of the glucose transporters. *Protein science : a publication of the Protein Society.* 2016;25(3):546-558.
118. Jentsch TJ. VRACs and other ion channels and transporters in the regulation of cell volume and beyond. *Nature reviews. Molecular cell biology.* 2016.

119. Abdelhady S, Kitambi SS, Lundin V, et al. Erg channel is critical in controlling cell volume during cell cycle in embryonic stem cells. *PloS one.* 2013;8(8):e72409.
120. Girault A, Brochiero E. Evidence of K+ channel function in epithelial cell migration, proliferation, and repair. *American journal of physiology. Cell physiology.* 2014;306(4):C307-319.
121. Francis KR, Wei L, Yu SP. SRC tyrosine kinases regulate neuronal differentiation of mouse embryonic stem cells via modulation of voltage-gated sodium channel activity. *Neurochemical research.* 2015;40(4):674-687.
122. Leitz J, Kavalali ET. Ca2+ Dependence of Synaptic Vesicle Endocytosis. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry.* 2015.
123. Hao B, Webb SE, Miller AL, Yue J. The role of Ca signaling on the self-renewal and neural differentiation of embryonic stem cells (ESCs). *Cell calcium.* 2016.
124. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. *Nature reviews. Molecular cell biology.* 2003;4(7):552-565.
125. Cain SM, Snutch TP. Contributions of T-type calcium channel isoforms to neuronal firing. *Channels (Austin, Tex.).* 2010;4(6):475-482.
126. Ko YH, Hong S, Pedersen PL. Chemical mechanism of ATP synthase. Magnesium plays a pivotal role in formation of the transition state where ATP is synthesized from ADP and inorganic phosphate. *The Journal of biological chemistry.* 1999;274(41):28853-28856.
127. Pantopoulos K, Porwal SK, Tartakoff A, Devireddy L. Mechanisms of mammalian iron homeostasis. *Biochemistry.* 2012;51(29):5705-5724.
128. Abreu IA, Cabelli DE. Superoxide dismutases-a review of the metal-associated mechanistic variations. *Biochimica et biophysica acta.* 2010;1804(2):263-274.
129. Tapiero H, Tew KD. Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie.* 2003;57(9):399-411.
130. Rodriguez JP, Rios S, Gonzalez M. Modulation of the proliferation and differentiation of human mesenchymal stem cells by copper. *Journal of cellular biochemistry.* 2002;85(1):92-100.
131. Hu J, Yang Z, Wang J, et al. Zinc Chloride Transiently Maintains Mouse Embryonic Stem Cell Pluripotency by Activating Stat3 Signaling. *PloS one.* 2016;11(2):e0148994.
132. Tokuoka S, Morioka H. The membrane potential of the human cancer and related cells. I. *Gan.* 1957;48(4):353-354.
133. Cone CD, Jr. Electroosmotic interactions accompanying mitosis initation in sarcoma cells in vitro. *Transactions of the New York Academy of Sciences.* 1969;31(4):404-427.
134. Cone CD, Jr., Tongier M, Jr. Control of somatic cell mitosis by simulated changes in the transmembrane potential level. *Oncology.* 1971;25(2):168-182.
135. Cone CD, Jr., Cone CM. Induction of mitosis in mature neurons in central nervous system by sustained depolarization. *Science (New York, N.Y.).* 1976;192(4235):155-158.

136. Deng XL, Lau CP, Lai K, Cheung KF, Lau GK, Li GR. Cell cycle-dependent expression of potassium channels and cell proliferation in rat mesenchymal stem cells from bone marrow. *Cell proliferation.* 2007;40(5):656-670.
137. Urrego D, Movsisyan N, Ufartes R, Pardo LA. Periodic expression of Kv10.1 driven by pRb/E2F1 contributes to G2/M progression of cancer and non-transformed cells. *Cell cycle (Georgetown, Tex.).* 2016;15(6):799-811.
138. Wu J, Zhong D, Fu X, Liu Q, Kang L, Ding Z. Silencing of Ether a go-go 1 by shRNA inhibits osteosarcoma growth and cell cycle progression. *International journal of molecular sciences.* 2014;15(4):5570-5581.
139. Wu J, Chen Z, Liu Q, Zeng W, Wu X, Lin B. Silencing of Kv1.5 Gene Inhibits Proliferation and Induces Apoptosis of Osteosarcoma Cells. *International journal of molecular sciences.* 2015;16(11):26914-26926.
140. Zhao Y, Wei H, Kong G, Shim W, Zhang G. Hydrogen sulfide augments the proliferation and survival of human induced pluripotent stem cell-derived mesenchymal stromal cells through inhibition of BKCa. *Cytotherapy.* 2013;15(11):1395-1405.
141. Borowiec AS, Bidaux G, Pigat N, Goffin V, Bernichtein S, Capiod T. Calcium channels, external calcium concentration and cell proliferation. *European journal of pharmacology.* 2014;739:19-25.
142. Nilius B. Chloride channels go cell cycling. *The Journal of physiology.* 2001;532(Pt 3):581.
143. da Veiga Moreira J, Peres S, Steyaert JM, et al. Cell cycle progression is regulated by intertwined redox oscillators. *Theoretical biology & medical modelling.* 2015;12:10.
144. Andang M, Hjerling-Leffler J, Moliner A, et al. Histone H2AX-dependent GABA(A) receptor regulation of stem cell proliferation. *Nature.* 2008;451(7177):460-464.
145. Fernando RN, Eleuteri B, Abdelhady S, Nussenzweig A, Andang M, Ernfors P. Cell cycle restriction by histone H2AX limits proliferation of adult neural stem cells. *Proceedings of the National Academy of Sciences of the United States of America.* 2011;108(14):5837-5842.
146. Rodriguez-Gomez JA, Levitsky KL, Lopez-Barneo J. T-type Ca2+ channels in mouse embryonic stem cells: modulation during cell cycle and contribution to self-renewal. *American journal of physiology. Cell physiology.* 2012;302(3):C494-504.
147. Vegara-Meseguer JM, Perez-Sanchez H, Araujo R, Martin F, Soria B. L-Type Ca(2+) Channels and SK Channels in Mouse Embryonic Stem Cells and Their Contribution to Cell Proliferation. *The Journal of membrane biology.* 2015;248(4):671-682.
148. Nguemo F, Fleischmann BK, Gupta MK, et al. The L-type Ca2+ channels blocker nifedipine represses mesodermal fate determination in murine embryonic stem cells. *PloS one.* 2013;8(1):e53407.
149. Ng SY, Chin CH, Lau YT, et al. Role of voltage-gated potassium channels in the fate determination of embryonic stem cells. *Journal of cellular physiology.* 2010;224(1):165-177.
150. Lau YT, Wong CK, Luo J, et al. Effects of hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blockers on the proliferation and cell cycle

progression of embryonic stem cells. *Pflugers Archiv : European journal of physiology.* 2011;461(1):191-202.
151. Stroh A, Tsai HC, Wang LP, et al. Tracking stem cell differentiation in the setting of automated optogenetic stimulation. *Stem cells (Dayton, Ohio).* 2011;29(1):78-88.
152. Kleger A, Seufferlein T, Malan D, et al. Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells. *Circulation.* 2010;122(18):1823-1836.
153. D'Ascenzo M, Piacentini R, Casalbore P, et al. Role of L-type Ca2+ channels in neural stem/progenitor cell differentiation. *The European journal of neuroscience.* 2006;23(4):935-944.
154. Sachinidis A, Schwengberg S, Hippler-Altenburg R, et al. Identification of small signalling molecules promoting cardiac-specific differentiation of mouse embryonic stem cells. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2006;18(6):303-314.
155. Lithium salts in the treatment of psychotic excitement: John F Cade. *The Australian and New Zealand journal of psychiatry.* 1982;16(3):129-133.
156. Moore GJ, Cortese BM, Glitz DA, et al. A longitudinal study of the effects of lithium treatment on prefrontal and subgenual prefrontal gray matter volume in treatment-responsive bipolar disorder patients. *The Journal of clinical psychiatry.* 2009;70(5):699-705.
157. Kara N, Narayanan S, Belmaker RH, Einat H, Vaidya VA, Agam G. Chronic Lithium Treatment Enhances the Number of Quiescent Neural Progenitors but Not the Number of DCX-Positive Immature Neurons. *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP).* 2015;18(7):pyv003.
158. Chen G, Rajkowska G, Du F, Seraji-Bozorgzad N, Manji HK. Enhancement of hippocampal neurogenesis by lithium. *Journal of neurochemistry.* 2000;75(4):1729-1734.
159. Focosi D, Azzara A, Kast RE, Carulli G, Petrini M. Lithium and hematology: established and proposed uses. *Journal of leukocyte biology.* 2009;85(1):20-28.
160. Satija NK, Sharma D, Afrin F, Tripathi RP, Gangenahalli G. High throughput transcriptome profiling of lithium stimulated human mesenchymal stem cells reveals priming towards osteoblastic lineage. *PloS one.* 2013;8(1):e55769.
161. Nora JJ, Nora AH, Toews WH. Letter: Lithium, Ebstein's anomaly, and other congenital heart defects. *Lancet (London, England).* 1974;2(7880):594-595.
162. Diav-Citrin O, Shechtman S, Tahover E, et al. Pregnancy outcome following in utero exposure to lithium: a prospective, comparative, observational study. *The American journal of psychiatry.* 2014;171(7):785-794.
163. Kao KR, Masui Y, Elinson RP. Lithium-induced respecification of pattern in Xenopus laevis embryos. *Nature.* 1986;322(6077):371-373.
164. Rogers I, Varmuza S. LiCl disrupts axial development in mouse but does not act through the beta-catenin/Lef-1 pathway. *Molecular reproduction and development.* 2000;55(4):387-392.

165. Stachel SE, Grunwald DJ, Myers PZ. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development (Cambridge, England).* 1993;117(4):1261-1274.
166. Hallcher LM, Sherman WR. The effects of lithium ion and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *The Journal of biological chemistry.* 1980;255(22):10896-10901.
167. Inhorn RC, Majerus PW. Properties of inositol polyphosphate 1-phosphatase. *The Journal of biological chemistry.* 1988;263(28):14559-14565.
168. Agam G, Bersudsky Y, Berry GT, Moechars D, Lavi-Avnon Y, Belmaker RH. Knockout mice in understanding the mechanism of action of lithium. *Biochemical Society transactions.* 2009;37(Pt 5):1121-1125.
169. van Calker D, Belmaker RH. The high affinity inositol transport system--implications for the pathophysiology and treatment of bipolar disorder. *Bipolar disorders.* 2000;2(2):102-107.
170. Ryves WJ, Harwood AJ. Lithium inhibits glycogen synthase kinase-3 by competition for magnesium. *Biochemical and biophysical research communications.* 2001;280(3):720-725.
171. Rhyu GI, Ray WJ, Jr., Markley JL. Enzyme-bound intermediates in the conversion of glucose 1-phosphate to glucose 6-phosphate by phosphoglucomutase. Phosphorus NMR studies. *Biochemistry.* 1984;23(2):252-260.
172. Spiegelberg BD, Xiong JP, Smith JJ, Gu RF, York JD. Cloning and characterization of a mammalian lithium-sensitive bisphosphate 3'-nucleotidase inhibited by inositol 1,4-bisphosphate. *The Journal of biological chemistry.* 1999;274(19):13619-13628.
173. York JD, Ponder JW, Majerus PW. Definition of a metal-dependent/Li(+)-inhibited phosphomonoesterase protein family based upon a conserved three-dimensional core structure. *Proceedings of the National Academy of Sciences of the United States of America.* 1995;92(11):5149-5153.
174. Ray WJ, Jr., Post CB, Puvathingal JM. Comparison of rate constants for (PO3-) transfer by the Mg(II), Cd(II), and Li(I) forms of phosphoglucomutase. *Biochemistry.* 1989;28(2):559-569.
175. Souza Ade A, da Silva GS, Velez BS, Santoro AB, Montero-Lomeli M. Glycogen synthesis in brain and astrocytes is inhibited by chronic lithium treatment. *Neuroscience letters.* 2010;482(2):128-132.
176. Rodriguez-Gil JE, Fernandez-Novell JM, Barbera A, Guinovart JJ. Lithium's effects on rat liver glucose metabolism in vivo. *Archives of biochemistry and biophysics.* 2000;375(2):377-384.
177. Plenge P. Lithium effects on rat brain glucose metabolism in long-term lithium-treated rats studied in vivo. *Psychopharmacology.* 1978;58(3):317-322.
178. Nattel S, Carlsson L. Innovative approaches to anti-arrhythmic drug therapy. *Nature reviews. Drug discovery.* 2006;5(12):1034-1049.
179. Wahl-Schott C, Biel M. HCN channels: structure, cellular regulation and physiological function. *Cellular and molecular life sciences : CMLS.* 2009;66(3):470-494.
180. Maher MP, Wu NT, Guo HQ, Dubin AE, Chaplan SR, Wickenden AD. HCN channels as targets for drug discovery. *Combinatorial chemistry & high throughput screening.* 2009;12(1):64-72.

181. Wilkars W, Wollberg J, Mohr E, et al. Nedd4-2 regulates surface expression and may affect N-glycosylation of hyperpolarization-activated cyclic nucleotide-gated (HCN)-1 channels. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 2014;28(5):2177-2190.
182. Williams AD, Jung S, Poolos NP. Protein kinase C bidirectionally modulates Ih and hyperpolarization-activated cyclic nucleotide-gated (HCN) channel surface expression in hippocampal pyramidal neurons. *The Journal of physiology.* 2015;593(13):2779-2792.
183. Noam Y, Ehrengruber MU, Koh A, et al. Filamin A promotes dynamin-dependent internalization of hyperpolarization-activated cyclic nucleotide-gated type 1 (HCN1) channels and restricts Ih in hippocampal neurons. *The Journal of biological chemistry.* 2014;289(9):5889-5903.
184. Lewis AS, Vaidya SP, Blaiss CA, et al. Deletion of the hyperpolarization-activated cyclic nucleotide-gated channel auxiliary subunit TRIP8b impairs hippocampal Ih localization and function and promotes antidepressant behavior in mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2011;31(20):7424-7440.
185. Cao-Ehlker X, Zong X, Hammelmann V, et al. Up-regulation of hyperpolarization-activated cyclic nucleotide-gated channel 3 (HCN3) by specific interaction with K+ channel tetramerization domain-containing protein 3 (KCTD3). *The Journal of biological chemistry.* 2013;288(11):7580-7589.
186. Ying SW, Tibbs GR, Picollo A, et al. PIP2-mediated HCN3 channel gating is crucial for rhythmic burst firing in thalamic intergeniculate leaflet neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience.* 2011;31(28):10412-10423.
187. Biel M, Wahl-Schott C, Michalakis S, Zong X. Hyperpolarization-activated cation channels: from genes to function. *Physiological reviews.* 2009;89(3):847-885.
188. He C, Chen F, Li B, Hu Z. Neurophysiology of HCN channels: from cellular functions to multiple regulations. *Progress in neurobiology.* 2014;112:1-23.
189. Norberg E, Karlsson M, Korenovska O, et al. Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis. *The EMBO journal.* 2010;29(22):3869-3878.
190. Satoh TO, Yamada M. A bradycardiac agent ZD7288 blocks the hyperpolarization-activated current (I(h)) in retinal rod photoreceptors. *Neuropharmacology.* 2000;39(7):1284-1291.
191. BoSmith RE, Briggs I, Sturgess NC. Inhibitory actions of ZENECA ZD7288 on whole-cell hyperpolarization activated inward current (If) in guinea-pig dissociated sinoatrial node cells. *British journal of pharmacology.* 1993;110(1):343-349.
192. Wu X, Liao L, Liu X, Luo F, Yang T, Li C. Is ZD7288 a selective blocker of hyperpolarization-activated cyclic nucleotide-gated channel currents? *Channels (Austin, Tex.).* 2012;6(6):438-442.
193. Han Y, Lyman K, Clutter M, et al. Identification of Small-Molecule Inhibitors of Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels. *Journal of biomolecular screening.* 2015;20(9):1124-1131.

194. Yazlovitskaya EM, Edwards E, Thotala D, et al. Lithium treatment prevents neurocognitive deficit resulting from cranial irradiation. *Cancer research.* 2006;66(23):11179-11186.
195. Kao GD, Jiang Z, Fernandes AM, Gupta AK, Maity A. Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. *The Journal of biological chemistry.* 2007;282(29):21206-21212.
196. Li B, Zhao H, Rybak P, Dobrucki JW, Darzynkiewicz Z, Kimmel M. Different rates of DNA replication at early versus late S-phase sections: multiscale modeling of stochastic events related to DNA content/EdU (5-ethynyl-2'deoxyuridine) incorporation distributions. *Cytometry. Part A : the journal of the International Society for Analytical Cytology.* 2014;85(9):785-797.
197. Hindley C, Philpott A. The cell cycle and pluripotency. *The Biochemical journal.* 2013;451(2):135-143.
198. Codega P, Silva-Vargas V, Paul A, et al. Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche. *Neuron.* 2014;82(3):545-559.
199. Janich P, Pascual G, Merlos-Suarez A, et al. The circadian molecular clock creates epidermal stem cell heterogeneity. *Nature.* 2011;480(7376):209-214.
200. Bouchard-Cannon P, Mendoza-Viveros L, Yuen A, Kaern M, Cheng HY. The circadian molecular clock regulates adult hippocampal neurogenesis by controlling the timing of cell-cycle entry and exit. *Cell reports.* 2013;5(4):961-973.
201. Schnell A, Chappuis S, Schmutz I, et al. The nuclear receptor REV-ERBalpha regulates Fabp7 and modulates adult hippocampal neurogenesis. *PloS one.* 2014;9(6):e99883.
202. Yin L, Wang J, Klein PS, Lazar MA. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. *Science (New York, N.Y.).* 2006;311(5763):1002-1005.
203. Nikolaeva S, Ansermet C, Centeno G, et al. Nephron-Specific Deletion of Circadian Clock Gene Bmal1 Alters the Plasma and Renal Metabolome and Impairs Drug Disposition. *Journal of the American Society of Nephrology : JASN.* 2016.
204. Collins I, Garrett MD. Targeting the cell division cycle in cancer: CDK and cell cycle checkpoint kinase inhibitors. *Current opinion in pharmacology.* 2005;5(4):366-373.
205. Gery S, Koeffler HP. The role of circadian regulation in cancer. *Cold Spring Harbor symposia on quantitative biology.* 2007;72:459-464.
206. Sun Y, Shi Z, Lian H, Cai P. Energy metabolic dysfunction as a carcinogenic factor in cancer cells. *Clinical and translational medicine.* 2016;5(1):14.
207. Hodge BA, Wen Y, Riley LA, et al. The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle. *Skeletal muscle.* 2015;5:17.
208. Chell JM, Brand AH. Nutrition-responsive glia control exit of neural stem cells from quiescence. *Cell.* 2010;143(7):1161-1173.
209. Gurumurthy S, Xie SZ, Alagesan B, et al. The Lkb1 metabolic sensor maintains haematopoietic stem cell survival. *Nature.* 2010;468(7324):659-663.

210. Scognamiglio R, Cabezas-Wallscheid N, Thier MC, et al. Myc Depletion Induces a Pluripotent Dormant State Mimicking Diapause. *Cell.* 2016;164(4):668-680.
211. Zhang J, Nuebel E, Daley GQ, Koehler CM, Teitell MA. Metabolic regulation in pluripotent stem cells during reprogramming and self-renewal. *Cell stem cell.* 2012;11(5):589-595.
212. Yagita K, Horie K, Koinuma S, et al. Development of the circadian oscillator during differentiation of mouse embryonic stem cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America.* 2010;107(8):3846-3851.
213. Shi S, Hida A, McGuinness OP, Wasserman DH, Yamazaki S, Johnson CH. Circadian clock gene Bmal1 is not essential; functional replacement with its paralog, Bmal2. Current biology : CB. 2010;20(4):316-321.