Universidade do Minho
Escola de Ciências da Saúde

Nuno Jorge Ramos Abreu da Silva Lamas

Harnessing the potential of pluripotent stem cells to develop novel platforms to study human motor neurons *in vitro*

janeiro de 2015

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Tese de Doutoramento em Medicina

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janeiro de 2015

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I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, ___/___/ ____.

Full name: _____

Signature: _____

The work presented in this Thesis was performed in the Jenifer Estess/Project A.L.S. Laboratory for Stem Cell Research at Columbia University Medical Center, New York City, NY, USA; and at Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS) of University of Minho, Braga, Portugal. This work was supported by the Portuguese Foundation for Science and Technology (FCT) through a PhD Studentship SFRH/BD/33421/2008; and also by the Luso-American Development Foundation (FLAD), Project ALS, P2ALS and NYSTEM.

Deus quere, o homen sonha, a obra nasce

God wills, man dreams, the task is born

Fernando Pessoa in Mensagem

SÍSIFO

Recomeça…

Se puderes,

Sem angústia e sem pressa.

E os passos que deres,

Nesse caminho duro

Do futuro,

Dá-os em liberdade.

Enquanto não alcances

Não descanses.

De nenhum fruto queiras só metade.

E, nunca saciado,

Vai colhendo

Ilusões sucessivas no pomar

E vendo

Acordado,

O logro da aventura.

És homem, não te esqueças!

Só é tua a loucura

Onde, com lucidez, te reconheças.

Miguel Torga in Diário XII

Aos meus pais, Ana Teresa, família e amigos

To my parents, Ana Teresa, family and friends

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ABSTRACT

Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have the capacity to differentiate into all three embryonic germ layers and give rise virtually to any cell type in the body. For this reason, they represent an exciting new approach to unravelling the mechanisms of human embryonic development, for drug discovery and disease modelling *in vitro*. The unique ability to generate relevant cell types from human pluripotent stem cells (hPSCs) opens the possibility of creating inexhaustible sources of cells that are otherwise not open to study in the human body, especially those from the central nervous system. In line with this, the successful specification of human motor neurons from hPSCs has opened new avenues for the study of motor neuron disorders like ALS, a fatal neurodegenerative disease characterized by progressive demise of motor neurons in the cortex, brainstem and anterior horn of the spinal cord. However, the motor neuron yields from existing differentiation protocols are suboptimal, leading to the generation of populations of mixed progenitor cells and postmitotic neurons. In addition, the current understanding on the survival requirements of human motor neurons remains limited. These are significant hurdles for the generation of neuronal cells in quantities that will allow the prosecution of innovative studies based on motor neurons generated from human pluripotent stem cells.

Here, we initially used spinal motor neuron cultures specified from hESCs following an optimized protocol and demonstrated a remarkably high level of ongoing birth of new motor neurons during the ensuing 15 days after the regular 31-day period of motor neuron differentiation. Based on previous rodent studies this finding was unexpected and could represent a significant potential confound for some published studies using cultures differentiated in similar conditions to reveal determinants that alter motor neuron survival. We envisioned taking advantage of the ongoing genesis of motor neurons in two distinct ways. On one hand, to address the problem of insufficient motor neuron yields, we conducted a low-throughput screening study by testing a small collection of 160 bioactive molecules to discover small molecules capable of increasing motor neuron numbers in culture, either by enhancing neurogenesis and/or increasing survival. The Rho-kinase (ROCK) inhibitor Y-27632 was the only tested compound shown to be capable of robustly increasing motor neuron numbers up to four-fold after 9 days in culture, an effect which was evident in both hESC- and hiPSC-derived motor neuron cultures. The increase in motor neuron numbers was later demonstrated to be associated with the enhancement of progenitor

proliferation and motor neuron survival. These effects were likely induced through an unknown ROCK-independent mechanism, since other seven small molecules in the family could not elicit comparable increases in motor neuron numbers.

On the other hand, to overcome the potential confound effect of ongoing neurogenesis on motor neuron survival studies we used FACS sorting to purify human motor neurons derived from a HB9::GFP hESC reporter line, based on their cellular characteristics and GFP expression. The resulting nearly pure populations of human motor neurons were used to create an assay for agents with direct effects on motor neuron survival. Y-27632 was successfully applied to expand hESC-derived motor neuron cultures before the FACS sorting procedures, leading to a final increase in total motor neuron yields. Similarly to previous studies employing purified populations of chick and rodent embryonic motor neurons, the purified human motor neurons were demonstrated to be responsive to the survival-promoting actions of specific neurotrophic factors (GDNF, BDNF and CNTF), as well as Y-27362 itself. Therefore, we successfully developed original strategies that allow us to significantly increase motor neuron yields from hPSC-derived cultures and to create a robust survival assay using a pure population of human motor neurons specified from hPSCs. Our findings reveal that Y-27632 might constitute a new powerful tool with invaluable contributions to the study of pluripotent stem-cell derived human motor neurons.

Human adipose tissue constitutes an appealing alternative source of stem cells which capture the genetic background of the person from which they are obtained. From fat tissue one can easily isolate adult human adipose-derived stem cells (hADSCs) which can be cultivated *in vitro* and differentiated to other cell types, though with a more restricted potential than hPSCs. The current knowledge on the survival and expansion requirements of hADSCs is limited and protocols to efficiently drive these cells towards a neuronal lineage still need to be developed. Here, we expanded the study of the effects of Y-27632 on human stem cells and were able to show that Y-27632 does not robustly increase the survival and proliferation of hADSCs, a novel finding which demonstrates that the effects of Y-27632 are likely to be cell-specific.

In summary, the different methodological advances reported in this thesis should be of general interest for the preparation of human motor neurons on a large scale and for studies addressing the molecular processes underlying motor neuron genesis, survival and degeneration. The body of knowledge reported in this thesis should be of general importance for researchers using human stem cells to study other neuronal populations and other diseases.

RESUMO

As células estaminais embrionárias humanas (hESCs) e as células estaminais pluripotentes induzidas humanas (hiPSCs) têm a capacidade de se diferenciar nos três folhetos germinativos embrionários e dar origem virtualmente a qualquer tipo celular existente no organismo. Dessa forma, constitutem uma nova abordagem na descoberta dos mecanismos que regulam o desenvolvimento embrionário humano, na descoberta de novos fármacos e na modelação de patologias *in vitro*. A capacidade única de gerar tipos celulares de interesse a partir destas células estaminais pluripotentes humanas (hPSCs) cria a possibilidade de serem geradas fontes ilimitadas de células que dificilmente estão acessíveis para estudo no corpo humano, sendo o caso das células do sistema nervoso central. Nesse sentido, a criação com sucesso de neurónios motores humanos a partir de hPSCs tem conduzido a novas avenidas no estudo de doenças do neurónio motor, sendo o caso da Esclerose Lateral Amiotrófica (ELA), uma doença neurodegenerative fatal caracterizada pela morte progressiva de neurónios motores no córtex cerebral, tronco cerebral e corno anterior da medula espinhal. No entanto, o resultado final dos protocolos de diferenciação existentes não são ainda os ideiais, dando origem à formação de populações mistas de células progenitoras neuronais e neurónios motores pós-mitóticos. Para além disso, o conhecimento sobre os requisitos destes neurónios motores para se manterem vivos é ainda escasso. Estes factos no seu conjunto constituem importantes barreiras para a produção de células neuronais em quantidades suficientes que permitam o desenvolvimento de estudos inovadores tendo por base os neurónios motores criados a partir de células estaminais pluripotentes humanas.

Neste trabalho, começamos inicialmente por usar culturas de neurónios motores da espinhal medula criados a partir de hESCs, seguindo um protocolo optimizado; e demonstramos níveis consideráveis de génese contínua de neurónios motores durante 15 dias após o período habitual de 31 dias para a diferenciação de neurónios motores. Tendo por base os estudos anteriores usando modelos animais, este achado não era expectável e constituía, de facto, um importante factor de confundimento em estudos onde eram utilizadas culturas de neurónios motores geradas em condições semelhantes para estudar factores que possam afectar a sobrevivência dos neurónios motores. Consideramos então que seria possível tirar partido da criação contínua de neurónios motores através de duas

abordagens distintas. Por um lado, no sentido de responder ao problema de produção de neurónios motores em quantidades insuficientes, conduzimos um teste de *screening* de moléculas em pequena escala, usando cerca de 160 compostos, para tentar encontrar alguma que fosse capaz de aumentar o número de neurónios motores em cultura, através da potenciação do efeito de neurogénese contínua e/ou aumento da sobrevivência neuronal. O inibidor da cinase Rho (ROCK) Y-27632 foi descrito como o único composto testado capaz de aumentar de forma significativa o número de neurónio motores em cultura, com efeito máximo de quadruplicação do número de neurónio motores ao fim de 9 dias em cultura, estando o efeito presente em culturas de neurónios motores geradas a partir de hESCs e hiPSCs. Este aumento no número de neurónios motores em cultura foi mais tarde demonstrado estar associado a uma potenciação da proliferação de células progenitoras, bem como, ao aumento da sobrevivência dos neurónios motores. Estes efeitos alegadamente ocorrem através de um mecanismo desconhecido, muito provavelmente independente da inibição de ROCK, na medida em que, outras sete moléculas da mesma família foram testadas e não foram capazes de originar aumentos semelhantes no número de neurónios motores.

Por outro lado, de forma a contornar o potencial efeito de confundimento nos estudos de sobrevivência dos neurónios motores inerente à neurogénese contínua, usamos a tecnologia FACS para isolar neurónios motores humanos criados a partir de uma linha reporter HB9::GFP, com base nas suas características celulares e expressão de GFP. As populações de neurónios motores praticamente puras resultantes foram então usadas para desenvolver um ensaio com o objectivo de testar agentes com efeito directo na sobrevivência dos neurónios motores humanos. Antes do procedimento de FACS, o composto Y-27632 foi usado com sucesso na expansão das culturas de neurónios motores, levando a números mais elevados de neurónios motores obtidos com este procedimento. Em linha com estudos anteriores envolvendo o uso de populações purificadas de neurónios motores embrionários de pinto e roedor, os neurónios motores humanos purificados neste trabalho demonstraram ter capacidade de responder às acções pró-sobrevivência de factores neurotróficos específicos (GDNF, BDNF e CNTF), bem como ao composto Y-27632. Deste modo, consegui desenvolver com sucesso algumas estratégias que permitem aumentar de forma significativa a produção de neurónios motores a partir de culturas derivadas de hPSCs, bem como, criar um ensaio de sobrevivência neuronal robusto, com

base em neurónios motores humanos purificados a partir de culturas de neurónios motores. Os nossos achados sugerem que o composto Y-27632 pode constituir uma nova ferramenta poderosa no estudo de neurónios motores humanos criados a partir de células estaminais pluripotentes.

O tecido adiposo constitui uma fonte alternativa interessante de células estaminais que retêm o património genético da pessoa de onde são obtidas. A partir da gordura podemos isolar com facilidade células estaminais humanas adultas derivadas da gordura (hADSCs), que podem ser cultivadas *in vitro* e se diferenciar noutros tipos celulares, apesar do potencial mais restrito quando comparadas com as hPSCs. O conhecimento actual sobre os requisitos de sobrevivência e expansão destas células é ainda limitado e protocolos para a geração eficiente de neurónios a partir destas ainda não foram desenvolvidos. Neste trabalho, ampliamos o estudo dos efeitos da molécula Y-27632 nas células estaminais humanas e fomos capazes de demonstrar que a aplicação do composto Y-27632 não aumenta a proliferação e sobrevivência das hADSCs, um novo achado que ajuda a demonstrar que os efeitos de Y-27632 serão provavelmente específicos consoante o tipo celular em estudo.

Em suma, os diferentes avanços metodológicos documentados nesta tese deverão ser de interesse geral na geração de neurónios motores humanos em grande quantidades, bem como, na condução de estudos com vista ao melhor conhecimento dos mecanismos da motoneurogénese, sobrevivência e degeneração. O conjunto de conhecimentos cristalizados nesta tese poderão vir a constituir-se como importantes para outros investigadores que usam células estaminais humanas para estudar outras populações neuronais e outras patologias.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

A4V – Alanine at codon 4 changed to valine

ALS – Amyotrophic Lateral Sclerosis

ALS2 – Alsin

BDNF – Brain-derived neurotrophic factor

bHLH – Basic helix-loop-helix

BMP – Bone morphogenic protein

C9ORF72 – Chromosome 9 open reading frame 72

cAMP – 3', 5' Adenosine monophosphate

c-Myc - Cellular myelocytomatosis oncogene

CNS – Central nervous system

CNTF – Ciliary neurotrophic factor

CRMP2 – Collapsin response mediator protein 2

D90A – Aspartic acid to alanine substitution in codon 90

DBX1 – Developing brain homeobox protein 1

DBX2 – Developing brain homeobox protein 2

DCTN1 – Dynactin

DMPK – Myotonic dystrophy kinase

EBs – Embryoid bodies

EMA – European Medicine Agency

EpiSC – Epiblast stem cell

FACS – Fluorescence activated cell sorting

fALS – Familial form of ALS

FBS – Fetal bovine serum

FDA – Food and Drug Administration

FGF – Fibroblast growth factor

FOXP1 – Forkhead box protein P1

FTLD – Frontotemporal lobar dementia

FUS – Fused in sarcoma

GDF15 – Growth differentiation factor 15

GDNF – Glial-derived neurotrophic factor

GFP – Green fluorescent protein

hADSC – Human adult adipose-derived stem cells

HB9 – Homeobox gene HB9

HD – Homeodomain

HESC – Human embryonic stem cell

hESC-MN – Human embryonic stem cell-derived motor neuron

hiPSC – Human induced pluripotent stem cells

HMC – Hypaxial motor column

HPSC – Human pluripotent stem cell

IGF-1 – Insulin-like growth factor 1

iPSC – Induced pluripotent stem cells

ISL1 – Islet 1

ISL2 – Islet 2

KLF4 - Kruppel-like factor 4

LHX1 – LIM/homeobox protein 1

LHX3 – LIM/homeobox protein 3

LIF – Leukemia inhibitory factor

LIMK – LIM kinase

LMC – Lateral motor column

LRRK2 – Leucine-rich repeat protein kinase-2

LRX3 – Leucine-rich repeat extensin-like protein 3

mESC – Mouse embryonic stem cell

mESC-MN – Mouse embryonic stem cell-derived motor neuron

MLC – Myosin light chain

MMC – Median motor column

MNK1 – MAP kinase interacting kinase 1

MRCK – Myotonic dystrophy kinase-related CDC42-binding kinase

MSK1 – Mitogen- and stress-activated protein kinase-1

NEAA – Non-essential aminoacids

NKX2.2 – NK2 homeobox 2

NKX6.1 – NK6 homeobox 1

NMJ – Neuromuscular junction

NT-3 – Neurotrophin 3

NT-4/5 – Neurotrophin 4/5

NT-ESC – Embryonic stem cells derived by nuclear transfer

OCT3/4 - Octamer 3/4

OLIG2 – Oligodendrocyte transcription factor 2

OPTN – Optineurin

PAX6 – Paired box protein 6

PAX7 – Paired box protein 7

PBS – Phosphate buffer saline

PFN1 – Profilin

PGC – Preganglionic motor column

PLS – Primary lateral sclerosis

PRK2 – Protein kinase C-related kinase 2

PTEN – Phosphatase and tensin homologue

RA – Retinoic acid

RALDH-2 – Retinaldehyde dehydrogenase-2

ROCK – Rho-associated kinase

sALS – Sporadic form of ALS

SCNT – Somatic cell nuclear transfer

SETX – Senataxin

SHH – Sonic hedgehog

SMA – Spinal Muscular Atrophy

SOD1 – Superoxide dismutase 1

SOX2 - Sex determining region Y-box 2

SQSTM1 – Sequestosome 1

SSEA – Stage-specific embryonic antigen

TDP-43 – TAR DNA binding protein

UBQLN2 – Ubiquilin 2

VAPB – Vesicle-associated membrane protein B

VCP – Valosin-containing protein

α – alpha

β – beta

γ – gamma

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Human pluripotent stem cells (hPSCs), through their capacity to differentiate into all three embryonic germ layers and give rise to virtually all cell types in the body, have opened unprecedented possibilities to generate *in vitro* nearly inexhaustible sources of cells which are normally inaccessible to study in the human body (Han et al., 2011; Nizzardo et al., 2010). This holds special promise in neurodegenerative disorders like amyotrophic lateral sclerosis (ALS) or spinal muscular atrophy (SMA), where healthy or patient-specific motor neurons are not available due to numerous ethical and technical constraints (Han et al., 2011; Nizzardo et al., 2010; Palmer et al., 2001; Silani et al., 1998). Thus, human embryonic and patient-specific induced pluripotent stem cells (hESCs and hiPSCs, respectively) represent a new potential powerful tool for studying human development, disease modeling and drug discovery (Daley and Scadden, 2008; Ebert and Svendsen, 2010a; Han et al., 2011; Lukovic et al., 2012; Marchetto and Gage, 2012; Mattis and Svendsen, 2011; Rubin and Haston, 2011).

1.1. The motor neuron

Motor neurons are specialized cells of the central nervous system (CNS) whose cell bodies are located in the motor cortex, in nuclei in the mid- and hind-brain and in columns that cover the full length of the ventral horns of the spinal cord (Elliott, 1945; Romanes, 1941, 1951). Motor neurons relay information from the brain to the periphery enabling the finely tuned contraction of muscles in the body (Grillner and Jessell, 2009; Kanning et al., 2010). In this manner, motor neurons are vital to the control of actions on which life depends, including swallowing and breathing (Grillner and Jessell, 2009; Jessell et al., 2011). The nearly 120,000 motor neurons in the spinal cord innervate the almost 300 bilateral pairs of muscles present in the human body (Kanning et al., 2010). In spite of their well-known shared functions, motor neurons constitute in fact a complex heterogeneous population of nerve cells (Kanning et al., 2010). From a clinical viewpoint, we can group the human motor neurons into upper and lower motor neurons. Upper motor neurons are situated in the motor region of the cerebral cortex and carry motor information to the appropriate level in the brain stem (corticobulbar tracts) and spinal cord (corticospinal tracts), from where nerve signals proceed to the muscles by means of the lower motor neurons, in some cases through

intervening spinal interneurons (Snell, 2010; Standring and Gray, 2008). The cortical motor neurons, known as Betz cells, are giant cells in the brain and are located in the layer V of the primary motor cortex (Rivara et al., 2003; Snell, 2010; Standring and Gray, 2008). Their axons project to the brainstem to form the lateral corticospinal tract on each side of the spinal cord, if they decussate and cross the midline; or the anterior corticospinal tracts, in the absence of decussation (Snell, 2010; Standring and Gray, 2008). Clinically, upper motor neuron lesions can cause paralysis, spasticity or hypertonicity, exaggerated deep tendon reflexes, an extensor plantar response known as Babinski's sign or absence of superficial abdominal and cremasteric reflexes (Snell, 2010; Standring and Gray, 2008). In contrast, lower motor neuron lesions lead to flaccid paralysis, atrophy, loss of reflexes in the afflicted areas, muscle fasciculation and contracture (Snell, 2010; Standring and Gray, 2008).

At the level of the spinal cord, which is the main focus of the current work, human motor neurons are grouped into distinct columns, divisions and pools (Arber, 2012; Dasen and Jessell, 2009; Dasen et al., 2003; Dasen et al., 2005; Routal and Pal, 1999). Such anatomical/functional organization is critical for the exquisitely coordinated contraction of the several muscle types in the body (Dalla Torre di Sanguinetto et al., 2008; Dasen and Jessell, 2009; Dasen et al., 2003). In terms of functionality, we can identify at least three main different subtypes of motor neurons, which are found at all levels of the spinal cord: alpha (α), gamma (γ) and beta (β) (Kanning et al., 2010). Alpha (α) motor neurons are the most abundant and drive muscle contraction by innervating extrafusal fibers (Hunt and Kuffler, 1951; Kanning et al., 2010; Kuffler et al., 1951). Gamma (γ) motor neurons innervate the intrafusal fibers of the muscle spindle and play complex roles in the control of the motor system by modulating the sensitivity of muscle spindles to stretch (Hunt and Kuffler, 1951; Kanning et al., 2010; Kuffler et al., 1951). Beta (β) motor neurons innervate both intra and extrafusal muscle and constitute a population of motor neurons which is less well studied and understood (Kanning et al., 2010). In terms of target innervation a fundamental distinction can be made: somatic motor neurons, the most abundant, which project their axons directly to the peripheral skeletal muscle (Dasen and Jessell, 2009); and the visceral preganglionic motor neurons, which synapse onto neuronal populations in the ganglia of the autonomic nervous system (sympathetic and parasympathetic), which in turn innervate cardiac and visceral smooth muscles (Dasen and Jessell, 2009). These visceral preganglionic motor neurons are clustered in a dorsal column called the preganglionic motor column (PGC)

and are found essentially at the thoracic levels (Dasen and Jessell, 2009; Prasad and Hollyday, 1991). On the other hand, somatic motor neurons are organized mainly into three distinct columns throughout the rostrocaudal length of the spinal cord (Dasen and Jessell, 2009). The median motor column (MMC) is a continuous column of somatic motor neurons in the medial-ventral aspect of the spinal cord whose motor neurons innervate the epaxial muscles of the dorsal body wall (Dasen and Jessell, 2009; Fetcho, 1987; Gutman et al., 1993). The hypaxial motor column (HMC) is located laterally to the MMC and is restricted to the thoracic spinal cord (Dasen and Jessell, 2009). Motor neurons in the HMC innervate intercostal and abdominal muscles of the ventral body wall (Dasen and Jessell, 2009; Gutman et al., 1993). Finally, the limb-innervating lateral motor columns (LMCs) are clusters of motor neurons located dorsally and laterally to the MMC and are found only at the level of the limbs (Dasen and Jessell, 2009). For these LMC columns a subsequent division into medial and lateral groups exists. Medial LMC motor neurons innervate muscles derived from the ventral primordial muscle of the limb, whereas the lateral LMC innervates the dorsal counterpart (Dasen and Jessell, 2009; Landmesser, 1978a, b). The clustering of motor neurons in these columns occurs during embryonic development due to a combination of factors expressed along the neural tube and transcriptional interactions which occur within those columns (Dasen and Jessell, 2009; Kanning et al., 2010). Each column is also characterized by the expression of a specific set of transcription factors: PGC motor neurons express Islet 1 (ISL1) and low levels of forkhead box protein P1 (FOXP1); HMC normally express ISL1, Islet 2 (ISL2) and ER81; MMC express ISL1, ISL2 and LIM/homeobox protein 3 (LHX3); whereas medial LMC motor neurons express ISL1, ISL2 and increased levels of FOXP1; and lateral LMC express ISL2, LIM/homeobox protein 1 (LHX1) and high levels of FOXP1 (Dasen and Jessell, 2009; Kanning et al., 2010). Within a given column, motor neurons are further organized into motor pools, which constitute clusters of motor neurons having similar molecular and morphological properties, which innervate a particular skeletal muscle (Dasen and Jessell, 2009). Therefore, an apparent correlation exists between the peripheral motor neuron targets and a hierarchical clustering of motor neurons in the spinal cord, characterized by the reproducible anatomical position of a given motor neuron group, as well its expression of a characteristic set of molecular markers (Arber, 2012; Dasen et al., 2008; Dasen and Jessell, 2009; Demireva et al., 2011; Livet et al., 2002; Surmeli et al., 2011).

1.2. Spinal motor neuron ontogeny in vertebrates

Embryonic development is characterized by a series of well-ordered events taking place with fine precision and which involve the accurate interaction of cells and molecules at a given spot and time in the embryo to allocate a particular cell fate (Davis-Dusenbery et al., 2014). The sequence of events leading to the generation of motor neurons in the spinal cord has started to be unraveled in the past couple of decades through numerous embryological studies and genetic analyses involving predominantly model organisms (Allodi and Hedlund, 2014; Briscoe and Ericson, 1999, 2001; Davis-Dusenbery et al., 2014; Jessell, 2000; Placzek and Briscoe, 2005). Prospective neural cells emerge initially at the neural plate level after neural induction (Jessell, 2000). Under the influence of extrinsic factors secreted by cells in the primitive streak of the gastrula and posterior paraxial mesoderm some future neural cells acquire a caudal character (Jessell, 2000). The mechanisms involved in the caudalization of these prospective neural cells are complex and comprise the concerted actions of fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), Wnts, retinoids and the so called caudalizing activity of the prospective paraxial mesoderm (Doniach, 1995; Jessell, 2000; Muhr et al., 1999). Indeed, the specification of neural cells of spinal cord identity seems to critically depend on the retinoids synthesized by the caudal paraxial mesoderm, which expresses specifically the enzyme retinaldehyde dehydrogenase-2 (RALDH-2) (Allodi and Hedlund, 2014; Jessell, 2000; Muhr et al., 1999).

The specification of motor neurons and all the other neuronal populations in the ventral neural tube is then determined by the graded expression of sonic hedgehog (SHH), which is released by the notochord and floor plate cells (Jessell, 2000; Roelink et al., 1995). Five distinctive domains of progenitor cells (p0, p1, p2, pMN, and p3) arise in the ventral aspect of the spinal cord due to a progressive ventral-high/dorsal-low SHH gradient (Briscoe and Ericson, 1999, 2001; Ericson et al., 1997; Jessell, 2000). From the p0-p3 progenitor domains four distinct classes of ventral interneurons are generated, whereas motor neurons arise from pMN neuronal progenitor cells which selectively express the oligodendrocyte transcription factor 2 (Olig2) (Briscoe and Ericson, 2001; Ligon et al., 2006; Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001). The exact mechanisms underlying the emergence of these unique cellular domains in response to graded SHH signals are not yet fully understood, but several experiments in the last couple of decades suggest a model where all these five progenitor domains are generated after the balanced expression of a

defined set of homeodomain (HD) proteins (Briscoe and Ericson, 2001; Jessell, 2000). Class II (HD) homeobox proteins NK2 homeobox 2 (Nkx2.2) and NK6 homeobox 1 (Nkx6.1) are induced by SHH, whereas class I HD proteins paired box protein 7 (Pax7), developing brain homeobox protein 1 (Dbx1,) developing brain homeobox protein 2 (Dbx2), leucine-rich repeat extensin-like protein 3 (Lrx3) and paired box protein 6 (Pax6) are repressed (Briscoe and Ericson, 2001; Jessell, 2000). In addition, each unique domain arises from a complex array of cross-repressive interactions between HD proteins expressed in adjacent domains, preventing the development of progenitor domains with hybrid identities and defining sharp boundaries between the domains (Briscoe and Ericson, 2001; Jessell, 2000).

The generation of motor neurons is restricted to the pMN progenitor domain due to the combined actions of the HD proteins Nkx6.1, Nkx2.2 and Lrx3 (Briscoe et al., 2000; Briscoe et al., 1999; McMahon, 2000; Sander et al., 2000). The activity of Nkx6.1, free from the opposing effects of Nkx2.2 and Lrx3, induces the expression of downstream factors which finally direct cells in this progenitor domain toward a motor neuron fate (Briscoe et al., 2000; Vallstedt et al., 2001). Among those factors, there is the homeodomain protein MNR2, whose expression is restricted to the pMN domain and which begins after the final cycle of division of the motor neuron progenitors starts (Briscoe and Ericson, 2001; Tanabe et al., 1998). This transcription factor, which positively regulates its own expression, induces downstream the production of the transcription factor set characteristically present in all recently born spinal motor neurons, namely homeobox gene HB9 (HB9), ISL1, ISL2 and LHX3 (Arber et al., 1999; Dasen and Jessell, 2009; Ericson et al., 1992; Pfaff et al., 1996; Sharma et al., 1998; Tanabe et al., 1998). The period of motor neuron production is relatively brief in rodents, occurring between embryonic days 9 and 11 (Arber et al., 1999; Nornes and Carry, 1978). Motor neurons are uninterruptedly produced from a short-lived pool of committed Olig2-positive progenitor cells, which are located ventrally in the spinal cord (Mukouyama et al., 2006).

After initially establishing this motor neuron progenitor domain, the transcription factor Olig2 later promotes progenitor cell cycle exit and neuronal differentiation (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou and Anderson, 2002). Following the critical period of motor neuron generation, the pool of motor neuron determining progenitors is exhausted or converted to oligodendroglial progenitors (Mukouyama et al., 2006). Apparently, there is an intrinsic loss of the potential to generate additional motor neurons beyond this exclusive

period, because Olig2 progenitor cells when isolated from late embryos and transplanted to the early neural tube were shown to produce only oligodendrocytes (Mukouyama et al., 2006). Indeed, later in development Olig2 has been demonstrated to drive the formation of oligodendrocyte precursors and mature oligodendrocytes (Fu et al., 2002; Lu et al., 2002; Zhou and Anderson, 2002; Zhou et al., 2001). The mechanisms underlying these unique temporal effects of the same transcription factor Olig2 during embryonic development only recently started to be unraveled (Gaber and Novitch, 2011; Li et al., 2011; Rabadan et al., 2012; Sun et al., 2011). Mounting evidence suggests that the activity of Olig2 during the development of the nervous system is regulated by distinct phosphorylation events (Gaber and Novitch, 2011; Yates, 2011). In the early embryo, Olig2 is phosphorylated at the residue serine-147, which leads to the formation of Olig2 homodimers that promote the expression of motor neuron-specific genes (Li et al., 2011). Later, there is dephosphorylation of serine-147, facilitating the transition to an oligodendrogenic fate by allowing Olig2 to form heterodimers with basic helix-loop-helix (bHLH) proteins that induce the expression of oligodendrocyte progenitor-specific genes and suppress neurogenesis (Li et al., 2011). In humans, the period of motor neuron production is considerably longer, and spans approximately twenty days from embryonic days 31 to 51 (Altman and Bayer, 2001; Bayer and Altman, 2002). Less is known about the molecular mechanisms underlying human motor neuron generation during embryonic development and whether similarly to rodents the progenitors in human motor neuron lineage are also pre-determined to generate limited numbers of motor neurons.

1.3. Newborn motor neurons rely on neurotrophic support for their survival

During the embryonic development of the vertebrate nervous system, neurons and glial cells are initially produced in excess and then undergo a process of massive naturally-occurring programmed cell death, which reduces cell numbers to nearly half of the number initially generated (Burek and Oppenheim, 1996; Buss et al., 2006; Henderson, 1996). Programmed cell death is particularly evident and well-documented in the embryonic spinal cord (Banker, 1982; Chu-Wang and Oppenheim, 1978; Hamburger, 1975; Henderson, 1996; Lance-Jones, 1982; O'Connor and Wyttenbach, 1974). For example, in the chick nearly 20,000 motor neurons are present in the spinal cord by embryonic day 5, but almost 8000 cells undergo programmed death by the end of the embryonic period (Hamburger, 1975;

Sendtner et al., 1996). Both pro-survival factors and pro-cell death pathways seem to be regulated in a finely orchestrated manner such that the magnitude of cell death is reproducible, not only in the spinal cord, but also across different neuronal populations (Burek and Oppenheim, 1996; Buss et al., 2006). Thus, programmed cell death might have evolved as a neuronal plasticity mechanism to exactly match the number of neurons and their targets (McLennan, 1982). Even though this suggests that a pre-defined genetic program is at the core of the naturally occurring cell death during spinal cord development, several classical experiments unequivocally demonstrated that spinal motor neurons that innervate the skeletal muscle critically depend on the presence of a target to correctly survive (Hamburger, 1958; Hollyday and Hamburger, 1976). Those pioneer experiments demonstrated that during the period of programmed motor neuron death, removal of target territories leads to enhanced demise of motor neurons, whereas the presence of an extra limb increases the pool of surviving motor neurons (Hamburger, 1958; Hollyday and Hamburger, 1976). Altogether, these data showed that target tissues are vital for the survival of motor neurons and implied that molecules derived from the target tissues act on motor neurons and render them resistant to cell death (Gould and Enomoto, 2009; Gould and Oppenheim, 2011). In support of this view, ensuing studies reported that extracts of purified muscle could significantly attenuate motor neuron cell death *in vitro* and *in vivo*, lending further support to the view that skeletal muscle mediated those effects on motor neuron survival (Calof and Reichardt, 1984; Dohrmann et al., 1986; Oppenheim et al., 1988). This landmark neurotrophic hypothesis was coined by Purves (Purves, 1988) and became the driving force for the subsequent quest initiated in the early 1990´s to find candidate motor neuron trophic factors (Gould and Enomoto, 2009). These are proteins capable of keeping motor neurons alive and were envisioned since the early days as tremendous therapeutic hopes for motor neuron degenerative disorders, which are characterized by motor neuron loss (Appel and Smith, 1993; Ekestern, 2004; Henderson, 1995; Henderson et al., 1993a; Henriques et al., 2010; Sendtner, 1996).

The initial discoveries relied on the reductionist method of adding a candidate neurotrophic factor to the medium of cultured motor neurons undergoing programmed cell death, or removing it from motor neurons maintained by muscle extract, counting surviving neurons over time to evaluate its effects on motor neuron survival (Bar, 2000; Gould and Oppenheim, 2011; Henderson et al., 1998; Sendtner et al., 2000). These *in vitro* motor

neuron survival assays using purified embryonic motor neurons from both chick and rodent spinal cord played a major role in the identification of several prospective neurotrophic factors (Bar, 2000; Henderson et al., 1998; Sendtner et al., 2000). From the early days of brain-derived neurotrophic factor (BDNF) (Henderson et al., 1993b; Koliatsos et al., 1993; Oppenheim et al., 1992; Sendtner et al., 1992; Yan et al., 1992), neurotrophin-3 (NT-3) (Henderson et al., 1993b; Li et al., 1994; Yan et al., 1993), neurotrophin-4/5 (NT-4/5) (Friedman et al., 1995; Hughes et al., 1993; Koliatsos et al., 1994; Li et al., 1994; Schmalbruch and Rosenthal, 1995), ciliary neurotrophic factor (CNTF) (Arakawa et al., 1990; Oppenheim et al., 1991; Sendtner et al., 1991; Sendtner et al., 1990), insulin-like growth factor 1 (IGF-1) (Ang et al., 1992; Lewis et al., 1993; Li et al., 1994; Neff et al., 1993) or glial-derived neurotrophic factor (GDNF) (Henderson et al., 1994; Li et al., 1995; Oppenheim et al., 1995; Yan et al., 1995; Zurn et al., 1994) identification up to the most recently discovered progranulin (Van Damme et al., 2008) and growth differentiation factor 15 (GDF-15) (Strelau et al., 2009), more than 20 proteins have been proposed as motor neuron trophic factors (Gould and Oppenheim, 2011; Kanning et al., 2010).

Most of these discoveries required the isolation of motor neurons from the complex environment of the spinal cord, an approach which allowed for the identification of factors that act directly on motor neurons, significantly facilitated direct quantification of motor neuron survival, and opened the door to a myriad of biochemical studies. These invaluable experiments would not have been possible if other cell types were present in the cultures; indeed without motor neuron purification, the field had previously struggled for many years to identify the neurotrophic factors. Although this might be considered a simplistic strategy, conclusions about both survival factors and cell death mechanisms were subsequently validated *in vivo*, demonstrating that the advantages of motor neuron purification prevail over concerns about the artificial nature of the assay. Interestingly, knockout mice for many of these factors or their receptors were demonstrated to have decreased total numbers of spinal motor neurons at the end of embryogenesis (Gould and Enomoto, 2009; Kanning et al., 2010). Yet, none of the knockout strains exhibited total motor neuron loss (Gould and Enomoto, 2009; Kanning et al., 2010). One possible explanation for this finding is that different motor neuron subtypes respond to a distinct neurotrophic factor or combination of neurotrophic factors, reflecting the heterogeneity of transcriptional identity, target innervation and physiological function discussed above (Henderson et al., 1998; Kanning et

al., 2010). However, for most neurotrophic factors the specific subtype of motor neurons on which they act on and specifically promote survival has not been identified (Kanning et al., 2010). One exception is GDNF, whose secretion by the muscle spindle has been demonstrated to be critical for the postnatal survival of gamma (γ) motor neurons (Shneider et al., 2009). GDNF was shown to be capable to regulate pool-specific, cell migration, axonal growth and synaptic connectivity by inducing PEA3-dependent transcriptional programs (Haase et al., 2002). Interestingly, the absence of GDNF in embryonic mice was shown to alter the location of developing spinal motor neurons which innervate the limbs (Haase et al., 2002; Kramer et al., 2006). Motor neurons in the postnatal period still depend on the surrounding cell types for neurotrophic support and recent work has shown that ablation of muscle cells leads to increases in spinal motor neuron death (Grieshammer et al., 1998; Holtmann et al., 2005; Kablar and Rudnicki, 1999; Phelan and Hollyday, 1991; Strelau et al., 2009). This suggests that neurotrophic factors from skeletal muscle continue to prevent the death of motor neurons normally expected to persist after the embryonic period (Gould and Oppenheim, 2011).

In humans, the survival requirements of motor neurons remain largely unexplored and the mechanisms responsible for motor neuron cell death remain to be systematically dissected, especially because human motor neurons are hard to obtain from patients and to grow appropriately *in vitro* (Nizzardo et al., 2010; Silani et al., 1998). This limitation can possibly be circumvented through the use of hPSCs to create human motor neurons. Those nervous cells created from hPSCs have been exposed to cocktails of neurotrophic factors to extend their survival *in vitro*, based on the knowledge gathered from the previous chick and rodent-based research. However, studies addressing the response of human motor neurons to individual neurotrophic factors have not been pursued. Thus, robust survival assays similar to the ones developed for chick and rodents using purified populations of motor neurons would help to gather important knowledge on human motor neuron survival needs and death-inducing mechanisms. Yet, assays with such characteristics have not been developed; standard protocols for hPSC differentiation generate mixed populations of spinal neurons of which motor neurons constitute a minority; and to date survival of purified human motor neurons has necessitated generally co-culture with other cell types. Therefore, there is a need to develop a robust survival assay using purified human motor neurons.

1.4. Neurodegenerative motor neuron disorders

The motor neuron disorders are a group of neurodegenerative conditions that selectively affect motor neurons, leading progressively to motor impairment and ultimately death (McDermott and Shaw, 2008; Talbot and Marsden, 2008; Talbot and Oxford University Press., 2010; Worms, 2001). Collectively, they can be divided into three categories: those with combined upper motor neuron and lower motor neuron involvement [e.g. amyotrophic lateral sclerosis (ALS)]; those with exclusive upper motor neuron degeneration [e.g. primary lateral sclerosis (PLS)] or those with specific lower motor neuron involvement [e.g. spinal muscular atrophy (SMA)] (McDermott and Shaw, 2008; Nizzardo et al., 2010; Talbot and Marsden, 2008).

The most common motor neuron degenerative disorder is ALS, also known as Lou Gehrig's disease, which was described in the scientific literature for the first time in 1869 by the French neurologist and anatomical pathologist Jean-Marie Charcot (Kiernan et al., 2011; Rowland, 2001). Amyotrophic lateral sclerosis is an invariably fatal disease characterized by progressive degeneration and death of motor neurons in the motor cortex, brainstem and anterior horn of the spinal cord (Kiernan et al., 2011; Leblond et al., 2014; Rowland and Shneider, 2001). Clinically, ALS normally presents as a progressive muscular weakness leading to paralysis and death (Kiernan et al., 2011; Wijesekera and Leigh, 2009). Generally, there is a delay of 1 to 2 years between the initial symptoms and the diagnosis of ALS, which is essentially clinical due to the lack of a specific diagnostic test (Ludolph, 2011; Rowland and Shneider, 2001). The time course of the disease can be markedly heterogeneous, however the prognosis is poor for all patients, most of whom die within 3 to 5 years after the initial diagnosis (Rowland and Shneider, 2001; Wijesekera and Leigh, 2009). The median survival after symptom onset for ALS patients is 27.5 months and the 4-year survival rate is around 40% (Hardiman et al., 2011; Su et al., 2014). Approximately 20% of patients survive longer than 5 years (Cooper-Knock et al., 2014). Nearly 10% of the patients are able to survive for more than 8 years (Hardiman et al., 2011; Kiernan et al., 2011).

The ALS phenotype is highly heterogeneous and the survival of patients is influenced by many factors, including the clinical phenotype, rate of disease progression, nutritional status and the specialized management of respiratory failure (Jenkins et al., 2014; Vucic et al., 2014). ALS affects women and men equally and the risk of the disease increases with age, with the average age of onset being 61.8 years (Chio et al., 2013; Orrell, 2007;

Rowland and Shneider, 2001). The incidence of this neurodegenerative disease is approximately 2 per 100,000 persons per year and the prevalence is approximately 6 per 100,000 persons (Chio et al., 2013; Dunckley et al., 2007; Orrell, 2007; Worms, 2001). The lifetime risk of developing the disease is 1:400 (Hardiman et al., 2011).

Typically, the disease process starts in one area and spreads in an anatomically contiguous manner throughout the motor system (Kanouchi et al., 2012; Ravits, 2014; Ravits and La Spada, 2009). Generally, this involves insidious progression of weakness starting in one limb or the bulbar muscles (Kanouchi et al., 2012; Ravits et al., 2007; Ravits and La Spada, 2009). In extremely rare cases, the disease starts simultaneously in multiple areas or in the respiratory muscles (Kanouchi et al., 2012; Ravits, 2014; Ravits and La Spada, 2009). Extensive research has shown that different motor neuron groups show a differential vulnerability to neurodegeneration in ALS (Kanning et al., 2010; Ravits et al., 2013; Saxena and Caroni, 2011). In fact, the large α motor neurons are the first to degenerate and fast-twitch motor units are preferentially affected in both ALS patients and mouse models (Gordon et al., 2010; Hegedus et al., 2007; Kanning et al., 2010; Pun et al., 2006). It is possible that the selective vulnerability of large motor neurons is linked to unmet higher energetic demands or the presence of enhanced oxidative stress (Schmitt et al., 2014). Interestingly, the motor neurons in the oculomotor nerves and those constituting the Onuf's nucleus are resistant to degeneration in ALS (Gizzi et al., 1992; Mannen et al., 1977, 1982; Schroder and Reske-Nielsen, 1984).

Another major hurdle in the field is the current lack of a definitive diagnostic test for ALS and, therefore, the diagnosis is mainly based on the presence of suggestive clinical signs coupled with negative laboratory tests and imaging studies for other differential diagnosis (Baumer et al., 2014; Vucic et al., 2014). To help in the diagnostic process and also to more accurately classify patients for research studies and clinical drug trials, increasing efforts have been undertaken in the last decades to unify patient symptoms and signs into a clinically well-defined entity (Wijesekera and Leigh, 2009). To this end, the Research Group on Motor Neuron Diseases from the World Federation of Neurology (WFN) developed the 1994 El Escorial diagnostic criteria and recently the Arlie House criteria (Brooks, 1994; Brooks et al., 2000; Hardiman et al., 2011).

The sporadic forms of the disease (sALS) are predominant, with only 5 to 10% of the cases having a familial genetic cause underlying (McDermott and Shaw, 2008; Rowland and

Shneider, 2001). These rare cases are called familial forms of ALS (fALS), resulting from highly penetrant monogenic mutations (Dunckley et al., 2007). Nonetheless, the clinical courses of fALS and sALS are virtually indistinguishable (Rowland and Shneider, 2001). Interestingly, in some cases thought to be sporadic one can also identify mutations in some of the genes known to be linked to the familial forms of the disease (Finsterer and Burgunder, 2014; Renton et al., 2014). The striking advances in genetics research over the past 20 years allowed the identification of mutations in more than 10 genes which seem to contribute to fALS onset, namely genes encoding the proteins superoxide dismutase 1 (SOD-1) (Rosen et al., 1993), alsin (ALS2) (Hadano et al., 2001), dynactin (DCTN1) (Puls et al., 2003), fused in sarcoma (FUS) (Kwiatkowski et al., 2009; Vance et al., 2009), optineurin (OPTN) (Maruyama et al., 2010), senataxin (SETX) (Chen et al., 2004), TAR DNA binding protein (TDP-43) (Sreedharan et al., 2008), ubiquilin 2 (UBQLN2) (Deng et al., 2011), vesicle-associated membrane protein B (VAPB) (Nishimura et al., 2004), profilin (PFN1) (Wu et al., 2012), valosin-containing protein (VCP) (Johnson et al., 2010), sequestosome 1 (SQSTM1) (Rubino et al., 2012; Teyssou et al., 2013) and Chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), among others (Andersen and Al-Chalabi, 2011; Leblond et al., 2014; Renton et al., 2014; Siddique and Ajroud-Driss, 2011).

In terms of fALS cases, nearly 12% show missense mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene on chromosome 21 (Renton et al., 2014; Wijesekera and Leigh, 2009). This was the first discovered pathogenic mutation associated with fALS (Rosen et al., 1993) and is the second most frequently identified cause of fALS (Renton et al., 2014). Genetic research over the past 20 years has allowed the identification of more than 170 mutations in the SOD1 gene, which are associated with diverse clinical phenotypes and courses (Su et al., 2014). For example, the D90A (aspartic acid to alanine substitution in codon 90) SOD1 mutation is recessive and patients homozygous for this SOD1 variant are mildly affected, with patient survival usually greater than 10 years (Andersen et al., 1996). In contrast, the A4V (alanine at codon 4 changed to valine) SOD1 dominant mutation causes a rapidly progressive form of ALS, with average survival of 1.4 years after symptoms onset (Juneja et al., 1997; Su et al., 2014). The discovery of SOD1 mutations in ALS led to the creation of the first animal and *in vitro* ALS disease models from which much of the current

knowledge on the disease has been gathered (McGoldrick et al., 2013; Turner and Talbot, 2008).

In 2011, a novel major breakthrough was reported in the field of ALS, with the identification of an expanded GGGGCC hexanucleotide repeat in C9ORF72, a newly identified predisposing factor to the sporadic forms of both ALS and frontotemporal lobar dementia (FTLD) (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The C9ORF72 gene is located on the short (p) arm of chromosome 9 open reading frame 72 and no known functions have been ascribed to the C9ORF72 protein yet (Cooper-Knock et al., 2014). The dominantly inherited hexanucleotide repeat expansion intronic to C9ORF72 appears to be the most common cause of fALS, frontotemporal lobar dementia (FTLD) and ALS–FTLD (Cooper-Knock et al., 2014; Majounie et al., 2012; Vucic et al., 2014). In fact, these hexanucleotide expansions are associated with nearly 40% of fALS cases and almost 20% of sporadic ALS cases (Vucic et al., 2014). The understanding of ALS pathogenesis increased significantly with the discovery of C9ORF72 hexanucleotide expansions, given that these expansions are linked not only to ALS, but also to FTLD; which suggests that ALS is a neurodegenerative disorder affecting multiple systems rather than the classical view of a pure neuromuscular disorder (Cooper-Knock et al., 2014; Turner et al., 2013). A recently proposed neuropathological hallmark of C9ORF72-associated ALS and FTLD is the accumulation in hippocampal and cerebellar neurons of TDP-43 (TAR DNA-binding protein 43, TARDBP) together with p62-positive TDP-43-negative inclusions (Al-Sarraj et al., 2011; Mann et al., 2013; Mori et al., 2013), hinting at the existence of a common pathophysiological pathway (Yokoyama et al., 2014). Interestingly, ALS and FTLD can now be proposed to join the exclusive club of expansion repeat disorders, a group of >22 inherited neurodegenerative diseases characterized by genomic expanded nucleotide repeat sequences (Ravits et al., 2013). The detailed mechanisms by which the C9ORF72 gene expansion leads to neurodegeneration in ALS remain elusive (Gendron et al., 2014), but current knowledge proposes a gain-of-function related to RNA toxicity as the most likely pathogenic mechanism; instead of loss-of-function of the associated protein (Cooper-Knock et al., 2014; Donnelly et al., 2013; Su et al., 2014).

The identification of ALS-linked mutations has permitted the generation of several animal and *in vitro* cellular models that have helped analyse the pathophysiological mechanisms contributing to ALS (Turner and Talbot, 2008). Nevertheless, an unifying model

of the molecular mechanisms accounting for motor neuron degeneration is still lacking (Ferraiuolo et al., 2011; Ravits et al., 2013; Turner et al., 2013). More importantly, no major therapeutic advances have been achieved in the last decades, meaning that ALS is still incurable (Aggarwal and Cudkowicz, 2008; Zinman and Cudkowicz, 2011). In fact, besides respiratory care and adequate nutritional support (Andersen et al., 2007; Diagnosis et al., 2012; Goyal and Mozaffar, 2014; Jenkins et al., 2014), riluzole is still the only medication approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) showing some benefits in survival and quality of life for people with ALS (Miller et al., 2007; Miller et al., 2012). However, recent reports show that riluzole may only slow disease progression and delay death by approximately 2 to 3 months (Miller et al., 2007; Miller et al., 2012). Therefore, the development of new disease-modifying therapeutic strategies for both fALS and sALS is paramount and certainly one of the most difficult challenges in the field.

1.5. Drug discovery using animal models of motor neuron disease has failed to translate into clinically applicable therapeutic strategies

Valid and reproducible disease models to unravel pathogenic mechanisms and evaluate the utility of new therapies are fundamental to the development of novel pharmacological agents to halt the progression or cure motor neuron diseases. The identification of several disease-linked mutations in ALS has enabled the generation of a myriad of *in vitro* and *in vivo* models, not only to study disease mechanisms, but also to test promising new therapies that might show benefits when treating humans (Lanka and Cudkowicz, 2008; Su et al., 2014; Turner and Talbot, 2008). Among these, mouse models overexpressing mutant forms of the human SOD1 protein have been the most widely studied animal models of ALS (Jackson et al., 2002; Pasinelli and Brown, 2006; Turner and Talbot, 2008; Van Den Bosch, 2011). Much knowledge has been gained from studying these mutant mice and they have also served as the best available benchmark preclinical platforms to test numerous promising drug candidates, including antiepileptic agents, antibiotics, anti-oxidants, anti-inflammatory drugs, anti-apoptotic small molecules, neurotrophic factors, protease inhibitors and genetic approaches (Lanka and Cudkowicz, 2008; Turner and Talbot, 2008; Vincent et al., 2008). Invariably, over the past decades, promising studies using those animal models have failed to demonstrate solid benefits in human clinical trials (Berry and

Cudkowicz, 2011; Ludolph and Sperfeld, 2005; Su et al., 2014; Turner and Talbot, 2008). There are several possible explanations for this apparent mismatch between promising animal data and the lack of effective translatable human therapies for motor neuron disease (Benatar, 2007; Lanka and Cudkowicz, 2008; Turner and Talbot, 2008; Vincent et al., 2008). For example, taking the transgenic mutant SOD1 mouse models, it remains to be determined whether those mouse models can truthfully mimic both fALS and sALS or whether they can only model fALS, especially when the great majority of those models are created through expression of abnormally high copy numbers of the mutated gene (Benatar, 2007; Turner and Talbot, 2008; van der Worp et al., 2010). Another important concern is that in most of the animal trials the treatments begin to be implemented before the clinical onset of the disease, a strategy which is impossible to follow in human clinical trials because relevant biological markers to identify patients at high risk of developing motor neuron disease have not been developed (Aggarwal and Cudkowicz, 2008; Benatar, 2007; Berry and Cudkowicz, 2011; Otto et al., 2012). Also to consider are questions regarding the numerous pharmacokinetic differences between mice and human, which makes it difficult to directly extrapolate to humans the dosage and pharmacokinetics of the mouse context (Benatar, 2007; Lanka and Cudkowicz, 2008). There is also a frequent mismatch in terms of outcome measures among animal and human studies (Benatar, 2007). Furthermore, a recent systematic analysis of nearly 100 published animal studies pointed out that the great majority of studies published until today besides being of questionable methodological quality, did not involve randomization and blindness to treatment, which are standard obligatory conditions for a well-conducted human clinical trial (Benatar, 2007; Vincent et al., 2008). In addition, errors in the design of clinical trials have also contributed to the current lack of success (Benatar, 2007; Berry and Cudkowicz, 2011).

A common mistake observed in phase III clinical trials is the presence of improper information on drug dosage and its biological activity in humans (Benatar, 2007; Cudkowicz et al., 2010). In fact, this is a major hurdle if one anticipates that using high doses of a given drug can lead to an enhanced rate of side effects and poor tolerability with consequent increase in the number of patients abandoning the studies (Lanka and Cudkowicz, 2008). On the other hand, dosages that are too low may fail to reach biological activity and lead to misleading conclusions about a promising therapy (Lanka and Cudkowicz, 2008). Another major problem faced by researchers when conducting clinical trials are the difficulties to

estimate the number of patients required to demonstrate a statistically significant effect in the trial, given that ALS evolves differently from patient to patient; and control the confounding effects of prescription and non-prescription drugs taken by these patients when undergoing a clinical trial (Berry and Cudkowicz, 2011; Cudkowicz et al., 2010; Lanka and Cudkowicz, 2008). The ability to generate relevant human neuronal cell types from stem cells might open unprecedented paths towards meaningful drug discovery to treat motor neurodegenerative disorders like ALS and SMA. It is therefore important to create robust protocols and strategies to generate, grow and study those stem cell-derived human neuronal cells *in vitro*. By studying human cells and performing meaningful drugs tests directly on human cell types one could shorten the time of translation of interesting basic research findings towards clinical applicable strategies (Ebert and Svendsen, 2010a; Engle and Puppala, 2013; Engle and Vincent, 2014).

1.6. Specification of spinal motor neurons from embryonic stem cells

Embryonic stem cells (ESCs) are a unique group of cells isolated from the inner cell mass (ICM) of developing blastocysts which exhibit distinctive properties of self-renewal (capacity to generate more stem cells) and pluripotency [the ability to differentiate intro all three embryonic germ layers (endoderm, ectoderm and mesoderm)] (Evans, 2005, 2011; Yu and Thomson, 2008; Zwaka and Thomson, 2005). These particular stem cells can give rise virtually to any cell type in the body, and have been viewed as an exciting new tool to unravel the mechanisms of developmental biology, and for drug discovery, disease modelling and possibly cell replacement therapies (Evans, 2011; Evans and Kaufman, 1981; Gokhale and Andrews, 2009; Lerou and Daley, 2005; Martin, 1981). In 1981, the first ESCs were derived from mouse embryos independently by two research teams, first at the University of Cambridge (Evans and Kaufman, 1981) and six months later at the University of California, San Francisco (Martin, 1981). Initial successful attempts to isolate and culture *in vitro* human embryonic stem cells (hESCs) were reported in 1994 by Ariff Bongso and collaborators at the National University Kent Ridge in Singapore (Bongso et al., 1994). Those researchers employed human fallopian tube cells as a medium to grow hESCs and were able to maintain their pluripotent state for only two passages (Bongso et al., 1994). In 1998, James Thomson and collaborators at the University of Wisconsin made a major

breakthrough in the field of stem cell biology, by reporting a novel robust strategy to isolate and culture human embryonic stem cells (hESCs) involving the usage of a mitotically inactive mouse embryonic fibroblast feeder layer to guarantee continuous undifferentiated proliferation (Thomson et al., 1998). To generate the hESC lines the inner cell mass was isolated from the blastocyst (Thomson et al., 1998). This procedure leads almost inevitably to the destruction of a fertilized human embryo, which has caused considerable ethical discussions (de Wert and Mummery, 2003; Engels, 2002; Gavrilov et al., 2009; Landry and Zucker, 2004; Taylor, 2011; Walters, 2004). Mouse ESCs and hESCs derive from different embryonic stages, but are considered fundamentally equivalent in their inexhaustible capacity to give rise to any cell type of the embryonic germ layers (endoderm, ectoderm and mesoderm). Interesting differences can be rapidly exposed when their characteristics are assessed more thoroughly, namely different colony morphologies, growth factor requirements for self-renewal and pluripotency maintenance, expression of surface markers, epigenetic profile and resistance to apoptosis upon single cell dissociation (Ginis et al., 2004; Gokhale and Andrews, 2009; Ohgushi and Sasai, 2011; Sato et al., 2003; Schnerch et al., 2010). While mESCs form clumps which are rounded and tight, the hESC colonies are flatter and looser and grow more slowly, with a population doubling time of nearly 36h (Friel et al., 2005). In terms of self-renewal, mESCs benefit from supplementation with leukemia inhibitory factor (LIF) to sustain undifferentiated growth, while hESCs do not respond to LIF and commonly need a feeder cell layer and supplementation with basic fibroblast growth factor (bFGF) to grow undifferentiated (Bongso and Richards, 2004; Reubinoff et al., 2000; Thomson et al., 1998). In terms of markers of pluripotency, both mESCs and hESCs share the expression of some common markers, namely octamer-binding transcription factor 4 (OCT4), NANOG and alkaline phosphatase; but differ in the expression of stage-specific embryonic antigens (SSEA), which are surface antigens used in the early days to characterize murine embryonic development (Draper et al., 2002; Henderson et al., 2002). For example, mESCs express SSEA-1 in the undifferentiated state and SSEA-3 and SSEA-4 as they differentiate, which is essentially the reverse of what has been characterized in hESCs (Draper et al., 2002; Friel et al., 2005; Henderson et al., 2002). Interestingly, recent studies have suggested that hESCs are more similar to the pluripotent epiblast stem cells (EpiSCs), obtained from later embryonic stage post-implantation epiblasts; than mESCs, which are obtained from pre-implantation blastocysts (Brons et al., 2007; Tesar et al., 2007). In fact, hESCs are also

obtained from early stage culture blastocysts, but it is conceivable that human blastocyst cells may proceed until the epiblast status from which EpiSCs are obtained (Nichols and Smith, 2009; Tesar et al., 2007). If the ultimate aim is to develop clinically translatable therapies, these differences between mESCs and hESCs highlight the importance of pursuing relevant studies using hESCs. Even though there are common mechanisms, the fundamental detailed downstream signalling pathways may differ significantly (Rao, 2004).

A decade ago, for the first time Hynek Wichterle and collaborators at Columbia University demonstrated that embryonic stem cells could be robustly directed towards a specific cell fate by applying previously well-described developmental mechanisms (Wichterle et al., 2002). These authors demonstrated that spinal motor neurons could be specified from mESCs using known spinal cord developmental cues retinoic acid (RA) and SHH (Wichterle et al., 2002), considerably increasing the yield over earlier methods that relied on RA alone (Renoncourt et al., 1998). In brief, after an initial period of expansion in pluripotency and self-renewal-maintaining culturing conditions mESCs were allowed to differentiate by growing as free-floating aggregates termed embryoid bodies (EBs) (Wichterle et al., 2002). Both RA and SHH were applied *in vitro* in a logical and defined temporal window to match the regular *in vivo* mouse motor neurogenic period (Wichterle et al., 2002). The retinoic acid was initially used to commit neuroectodermal cells towards a spinal cord identity (Wichterle et al., 2002). Then, SHH was used to drive the previously caudalized prospective progenitor cells towards a motor neuron lineage (Wichterle et al., 2002). After 7 days in culture, a population of neuronal cells positive for HB9, ISL and choline acetyltransferase (ChAT) was obtained (Wichterle et al., 2002). These mouse embryonic stem cell-derived motor neurons (mESC-MNs) shared many of the well-known molecular characteristics of spinal motor neurons and once transplanted to chick embryonic neural tube they were able to both incorporate in the ventral horn of the spinal cord and to project axons to muscle targets (Wichterle et al., 2002). Subsequent studies demonstrated that these mESC-MNs generated *in vitro* could recapitulate functional properties of embryonic motor neurons *in vivo* (Miles et al., 2004). In addition, the mESC-MNs were shown to express properly functioning receptors for excitatory and inhibitory neurotransmitters, they developed adequate electrophysiological properties to produce characteristic firing patterns and were also capable of establishing functional cholinergic synapses with C2C12 myotubes in culture (Miles et al., 2004).

All this work inspired the development of protocols to direct the differentiation of motor neurons from hESCs with the first successful attempts being reported in 2005 (Li et al., 2005; Singh Roy et al., 2005). Similarly to mESCs, the developmental cues RA and SHH were used to drive motor neuron specification, but the time required to generate human embryonic stem cell-derived motor neurons (hES-MNs) increased up to nearly 5 weeks (Li et al., 2005; Singh Roy et al., 2005). After this extended period of culture mixed populations of progenitor cells and cells expressing putative motor neuron markers HB9, ISL and ChAT was obtained (Li et al., 2005; Singh Roy et al., 2005). In line with the knowledge gathered from the mESC-MNs, the human embryonic stem cell-derived motor neurons (hESC-MNs) were shown to be electrophysiologically active and to develop functional synapses with muscle cells *in vitro* (Li et al., 2005; Singh Roy et al., 2005). Since these pioneer publications, several attempts to improve these protocols have been made, including more efficient strategies to induce neuralization in hESCs and chemical substitutes of the expensive growth factor SHH (Boulting et al., 2011; Chambers et al., 2009; Karumbayaram et al., 2009; Patani et al., 2011). Nevertheless, it is still not possible to consistently generate populations of neuronal cells from hESCs containing more than 40% spinal cord motor neurons. Remarkably, reliable retinoid-free strategies to induce motor neuron generation from hESCs have also been reported (Patani et al., 2011). Human motor neurons specified this way seem to assume a caudal, medial motor column identity (Patani et al., 2011), contrary to the cells generated using the previously described classical protocol, which generates motor neurons biased to a cervical-brachial phenotype (Patani et al., 2011).

The mechanisms underlying the specification of different subtypes of human motor neurons have only recently started being unraveled and much remains to be understood before we can robustly generate *in vitro* all the different subtypes of motor neurons present in the human spinal cord. Despite all these exciting methodological advances, large scale studies involving human spinal motor neurons are not yet practical to pursue, since major technical obstacles remain, including the suboptimal motor neuron yields from the existent differentiation protocols. In addition, the detailed mechanisms which underlie human motor neuron specification during embryonic development remain to be unraveled. Besides, it remains to be established if similarly to rodents (Mukouyama et al., 2006), the progenitors in human motor neuron lineage are also pre-determined to generate a limited number of motor neurons. It is also plausible that small molecules that enhance the proliferation of

progenitors in the motor neuron lineage might be used to increase the yield of human motor neurons generated from human pluripotent stem cells.

1.7. Human induced pluripotent stem cells have allowed the generation of patient-specific spinal motor neurons

The generation of individual-specific pluripotent stem cells has always been a major long-term goal of the stem cell scientific community due to the far-reaching therapeutic and regenerative possibilities of cells with such characteristics. First, personalized stem cells would lead to the creation of cells and tissues immunologically matched to the donor, decreasing theoretically the chances of rejection if regenerative strategies were attempted. Second, the ability to study any cell type with the genetic background of the donor opens the prospects of patient-specific *in vitro* studies of disease mechanisms and possibly patient-directed drug therapies, a more realistic goal for personalized medicine. One initial strategy researchers envisioned to create personalized pluripotent stem cells was somatic cell nuclear transfer (SCNT), the methodology employed by Ian Wilmut and colleagues to clone Dolly the sheep from adult mammary cells (Campbell et al., 1996; Schnieke et al., 1997). SCNT is a conceptually simple and experimentally demanding technique through which the nucleus of a somatic cell is inserted into a recipient oocyte from which the original genetic material has been removed (Niemann and Lucas-Hahn, 2012; Pan et al., 2012). The fused donor nucleus-recipient oocyte is then induced to start the normal process of embryonic development, which is entirely supported by factors existent in the recipient oocyte (Niemann and Lucas-Hahn, 2012; Pan et al., 2012). However, not only has SCNT been technically difficult to accomplish in humans, but also the widespread ethical concerns regarding human cloning have considerably reduced the efforts towards human SCNT over the years (Clausen, 2010; Hyun, 2011; Skene et al., 2009; Wilmut, 1998). The first successful attempts to robustly use SCNT to generate personalized pluripotent stem cells have been reported in the last three years (Chung et al., 2014; Noggle et al., 2011; Tachibana et al., 2013; Yamada et al., 2014). These pioneer studies demonstrated that human therapeutic cloning to create personalized pluripotent stem cells is now possible, not only using donor nucleus from healthy donors, but also using cells from patients, as shown with the generation of embryonic stem cells derived by nuclear transfer (NT-ESCs) from a Type I

diabetic patient (Yamada et al., 2014). Therefore, these new SCNT methods open novel promising avenues for the use of patient-specific stem cells to study disease and to develop personalized therapies.

While many researchers were dealing with the technical and ethical obstacles to working with hESCs and SCNT, others were actively trying to understand the mechanisms underlying self-renewal and maintenance of pluripotency in embryonic stem cells (Chen and Daley, 2008; Noggle et al., 2005; Palmqvist et al., 2005; Suzuki et al., 2006; Wang et al., 2006). The advances achieved in this field led to the breakthrough successful derivation of induced pluripotent stem cells (iPSC) from adult mouse fibroblasts by Yamanaka's group from Kyoto University, in 2006 (Takahashi and Yamanaka, 2006). After a screen to test 24 genes that were linked to pluripotency, this group of researchers demonstrated that the combined overexpression of only four of the 24 factors tested [octamer 3/4 (Oct3/4), sex determining region Y-box 2 (Sox2), kruppel-like factor 4 (Klf4) and cellular myelocytomatosis oncogene (c-Myc)] could revert an adult fibroblast into a stem-cell like state (Takahashi and Yamanaka, 2006). These induced pluripotent stem cells (iPSC) not only invalidated one of the central dogmas of cell biology, but also paved the way for the development of straightforward methods to generate the long-aimed-for personalized patient-specific stem cells (Takahashi and Yamanaka, 2006). This approach was rapidly transferred to human fibroblasts leading to the historic generation of human induced pluripotent stem cells (hiPSCs) in 2007 by two independent research teams (Takahashi et al., 2007; Yu et al., 2007). Whereas Yamanaka's group used the same methodology and reprogrammed human fibroblasts by applying the same four factors (Takahashi et al., 2007); the group of James Thomson reported the generation of hiPSCs by overexpressing OCT4, SOX2, NANOG and LIN28 using a lentiviral system (Yu et al., 2007). These two milestone reports started a new era in stem cell biology and motivated a myriad of follow-up studies. First, scientists aimed to demonstrate that other cell types as well could be reprogrammed using these methods (Robinton and Daley, 2012; Stadtfeld and Hochedlinger, 2010). Soon, hiPSCs were being generated from keratinocytes, hepatocytes, adipose-derived stem cells, peripheral blood, among others (Pan et al., 2012; Robinton and Daley, 2012; Stadtfeld and Hochedlinger, 2010). Second, since reprogramming methods were non-efficient and involved the usage of two oncogenic transcription factors (c-Myc and Klf4), in addition to viral transduction to overexpress the four transcription factors, numerous studies have also aimed to investigate

methods to generate hiPSC lines in a more efficient and safer manner (Gonzalez et al., 2011; Robinton and Daley, 2012). In the meantime, others have studied the mechanisms underlying reprogramming and in numerous other studies researchers have compared hESCs with hiPSCs to confirm or refute their biological equivalence, a question without definitive answers so far (Amabile and Meissner, 2009; Bilic and Izpisua Belmonte, 2012; Puri and Nagy, 2012; Robinton and Daley, 2012). Remarkably, the earlier published studies hinted that hiPSCs were similar to hESCs in terms of morphology, maintenance requirements, surface antigens, capacity to differentiate into cell types representative of the three embryonic germ layers, gene expression and also epigenetic status of pluripotent cell-specific genes (Mallon et al., 2014; Takahashi et al., 2007; Yu et al., 2007). However, the past 5 years have witnessed an exponential increase in the number of studies that report numerous differences between hiPSCs and *bona fide* hESCs, raising fundamental questions regarding the clinical relevance of hiPSCs and their clinical applicability in the future (Bilic and Izpisua Belmonte, 2012; Cahan and Daley, 2013; Christodoulou and Kotton, 2012; Narsinh et al., 2011). Predictably, the advent of human induced pluripotent stem cells (hiPSCs) was also accompanied by a scientific boom in disease-applied research, aiming to model different types of diseases *in vitro* (Grskovic et al., 2011; Han et al., 2011; Robinton and Daley, 2012; Tiscornia et al., 2011). Thus, numerous studies have been published demonstrating the ability to robustly generate hiPSCs from innumerable diseases and proposing novel and promising *in vitro* disease phenotypes (Grskovic et al., 2011; Han et al., 2011; Robinton and Daley, 2012). In terms of neurological disorders, hiPSCs have made possible the generation of different neuronal cell types carrying the constellation of genetic traits associated with neurodegeneration in a given individual, which otherwise would not be accessible. Thus, hiPSCs have enabled the study of early events in disease onset and other degenerative processes otherwise hard to observe in post-mortem tissues. Regarding motor neuron degenerative disorders, the landmark study of Dimos and colleagues reported in 2008 not only demonstrated for the first time the differentiation of motor neurons from hiPSCs, using a protocol that was nearly identical to the one previously described to generate hESC-MNs (Dimos et al., 2008); but also showed that this method could be applied with success to fibroblasts derived even from aged patients with ALS (Dimos et al., 2008). Accordingly, using the innovative reprogramming technology developed originally by Yamanaka's lab, these researchers were able to create patient-specific ALS-hiPSCs and to

differentiate *in vitro* human motor neurons capturing the genetic background of the original patient, which immediately raised the hope to create models of human motor neuron disease *in vitro* (Dimos et al., 2008). Since this pioneer study, researchers have reprogrammed fibroblasts from several patients with motor neuron degenerative disorders and aimed to demonstrate that motor neurons derived from those cells could exhibit a relevant phenotype *in vitro* (Chen et al., 2014; Ebert and Svendsen, 2010b; Ebert et al., 2009; Egawa et al., 2012; Grskovic et al., 2011; Kiskinis et al., 2014; Sareen et al., 2012; Wainger et al., 2014). Even though some prospective motor neuron phenotypes have already been proposed, much remains to be done before one can claim to have a robust *in vitro* model of human motor neuron disease from both sporadic and genetic forms of the diseases. In fact, we lack comprehensive knowledge on the survival requirements of human motor neurons. Moreover, it is also mandatory to clearly define cellular phenotypes *in vivo* and translate them precisely to the *in vitro* context. The current inefficient motor neuron differentiation protocols lead to suboptimal yields and this is a topic of major concern regarding the development of appropriate *in vitro* models of motor neuron diseases using hiPSCs. Ultimately, the absence of robust assays and other adequate platforms to study the relevant features of the *in vitro*-generated motor neurons has constituted a major obstacle for the advancement of the field.

1.8. Are human adult stem cells a reliable alternative source of patient-specific stem cells to study neurological disorders?

The generation of individual-specific cell types from stem cells has been a long term goal in the field of regenerative medicine given the unlimited possibilities regarding drug discovery, disease modelling *in vitro* and personalized regenerative therapies. Besides human pluripotent stem cells, an alternative strategy which has been explored to achieve the goal of personalized regenerative medicine involves the use of human adult stem cells, which retain the capacity for self-renewal and to differentiate into specific cell types (Verfaillie, 2002; Wagers and Weissman, 2004). However, when compared with human pluripotent stem cells, they are able to give rise to only a limited number of cell types and therefore exhibit a smaller differentiation potential (Roobrouck et al., 2008; Sohni and Verfaillie, 2011). Human adult or somatic stem cells can be found at different levels in the

organism and their fundamental function is to maintain and renew the different tissues where they are located (Roobrouck et al., 2008; Temple, 2001; Wagers, 2012). An adult stem cell type which has increasingly attracted the interest of the scientific community are the human adult adipose-derived stem cells (hADSCs), which constitute a population of adult stem cells easily isolated from body tissues containing fat, under local anesthesia with minimal patient discomfort (Gimble and Guilak, 2003; Gimble et al., 2007). Strategies for efficient culture of these stem cells have been explored, rendering the procedures for stem cell culture less complex over time (Gimble et al., 2013). Human ADSCs retain the capacity to self-renew and to differentiate into different cell types, namely adipocytes, osteoblasts, chondrocytes, myocytes, cardiomyocytes and neuron-like cells (Bunnell et al., 2008; Gimble et al., 2013; Gimble et al., 2007; Lindroos et al., 2011). Therefore, due to their differentiation potential, simplicity to isolate and ability to proliferate *in vitro*, the hADSCs have been considered an attractive alternative source of patient-specific stem cells (Aguena et al., 2012; Mizuno et al., 2012; Salgado et al., 2010). The generation of neuronal cells from hADSCs has already been reported (Ahmadi et al., 2012; Jang et al., 2010), including the specification of motor neuron-like cells (Liqing et al., 2011). However, it remains to be determined whether hADSCs can actually differentiate into fully functional neuronal relevant cell types (Franco Lambert et al., 2009; Ross and Verfaillie, 2008). In fact, the great majority of the studies relied on morphology and immunophenotyping to characterize the generated neuronal and glial cells; thus, failing to convincingly demonstrate that the human adipose stem cell-derived neuronal cells have fully functional features *in vitro* (Franco Lambert et al., 2009). In addition, the yields are low, which is possibly linked to the scarce knowledge on the cues that can efficiently drive human adult stem cells toward a neuronal fate. It is conceivable that one can get better knowledge on how to specify different neuronal cell types from hADSCs if we could gain a better insight on the mechanisms that regulate the proliferation, survival and differentiation of hADSCs. The advent of strategies to efficiently generate relevant neuronal cell types from these human adult stem cells could greatly expand the possibilities of using human stem cells towards personalized medicine strategies aimed at treating neurological disorders.

1.9. REFERENCES

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CHAPTER 2

AIMS

2. AIMS

Human pluripotent stem cells (hPSCs; hESCs or hiPSCs) have opened unprecedented opportunities to study previously inaccessible neuronal populations from healthy controls and patients. In the particular case of motor neuron diseases, hPSCs represent a promising platform through which to increase our understanding of the mechanisms involved in keeping human motor neurons alive. In addition, the possibility of studying human motor neurons that capture the genetic background of patients opens new avenues towards a more comprehensive understanding of motor neuron degeneration, newer and better diagnostic tools, and ultimately more effective therapeutic strategies. In order to conduct these fundamental studies large quantities of human motor neurons, and robust survival assays to study them, are needed. However, the motor neuron yields obtained from existing protocols are far from optimal and robust survival assays using populations of hPSC-derived spinal cord motor neurons have not been developed. A potential additional source of personalized neuronal cells are the human adipose-derived stem cells (hADSCs), easily obtained from fat containing human tissues. However, the current knowledge on factors regulating their survival, proliferation and commitment towards a neuronal lineage is scarce.

Therefore, the main aims of this thesis work were:

1. To devise strategies to increase the yield and abundance of spinal cord motor neurons generated from human pluripotent stem cell lines.

2. To create a robust and reliable *in vitro* survival assay to study human spinal cord motor neurons differentiated from pluripotent stem cells lines.

3. To study the response of human spinal motor neurons to neurotrophic factors identified for rodent spinal motor neurons.

4. To study factors that might affect the survival and proliferation of hADSCs.

CHAPTER 3

EXPERIMENTAL WORK

CHAPTER 3.1

Nuno Jorge Lamas, Bethany Johnson-Kerner, Laurent Roybon, Yoon A. Kim, Alejandro Garcia-Diaz, Hynek Wichterle and Christopher E. Henderson

Neurotrophic requirements of human motor neurons defined using amplified and purified stem cell-derived cultures

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Neurotrophic Requirements of Human Motor Neurons Defined Using Amplified and Purified Stem Cell-Derived Cultures

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Abstract

Human motor neurons derived from embryonic and induced pluripotent stem cells (hESCs and hiPSCs) are a potentially important tool for studying motor neuron survival and pathological cell death. However, their basic survival requirements remain poorly characterized. Here, we sought to optimize a robust survival assay and characterize their response to different neurotrophic factors. First, to increase motor neuron yield, we screened a small-molecule collection and found that the Rho-associated kinase (ROCK) inhibitor Y-27632 enhances motor neuron progenitor proliferation up to 4-fold in hESC and hiPSC cultures. Next, we FACS-purified motor neurons expressing the Hb9::GFP reporter from Y-27632-amplified embryoid bodies and cultured them in the presence of mitotic inhibitors to eliminate dividing progenitors. Survival of these purified motor neurons in the absence of any other cell type was strongly dependent on neurotrophic support. GDNF, BDNF and CNTF all showed potent survival effects (EC_{50} 1–2 pM). The number of surviving motor neurons was further enhanced in the presence of forskolin and IBMX, agents that increase endogenous cAMP levels. As a demonstration of the ability of the assay to detect novel neurotrophic agents, Y-27632 itself was found to support human motor neuron survival. Thus, purified human stem cell-derived motor neurons show survival requirements similar to those of primary rodent motor neurons and can be used for rigorous cell-based screening.

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Introduction

In vitro differentiation of specific cell types from human pluripotent stem cells (hPSCs) allows for molecular and functional analysis of cells that are otherwise inaccessible. This holds special promise in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), where ethical and technical constraints prevent access to human spinal motor neurons [1]. Using protocols based on normal developmental pathways, it has proven possible to generate spinal motor neurons from both mouse and human embryonic stem cells (ESCs) [2 6]. These are an important source of new mechanistic insights into the developmental requirements of wildtype motor neurons in both species. Moreover, successful specification of motor neurons from human induced pluripotent stem cells (hiPSCs) has opened novel avenues for mechanistic analysis of neuronal cell death and drug testing in motor neuron disease models [1,4 8]. Yet our knowledge of the survival requirements of human motor neurons remains limited.

Cultured motor neurons from rodent embryos served as the basis for identification of the neurotrophic factors responsible for keeping motor neurons alive during development [9 11] and the same factors significantly retard motor neuron death in animal models of ALS [12]. In parallel, motor neurons cultured from mouse models of ALS shed light on the mechanisms underlying neurodegeneration [13]. All these discoveries required the purification of motor neurons from the complex environment of the spinal cord. This approach allowed for identification of factors

that act directly on motor neurons, significantly facilitated direct quantification of motor neuron survival, and opened the door to biochemical studies that would not have been possible in mixed cultures. Although this might be considered a reductionist approach, conclusions about both survival factors and cell death mechanisms were subsequently validated *in vivo* [14 21], demonstrating that the advantages of motor neuron purification outweigh concerns about the artificial nature of the assay. It is therefore important to extend such approaches to human motor neurons. However, standard protocols for hPSC differentiation generate mixed populations of spinal neurons of which motor neurons constitute a minority, and to date survival of purified motor neurons has necessitated generally co-culture with other cell types [22 25]. There is consequently a need for a robust survival assay based on purified human motor neurons.

Another challenge is that absolute numbers of motor neurons generated from hESC/hiPSCs by standard procedures are relatively low. During embryonic development in rodents, motor neurons are produced from a short-lived pool of committed ventral spinal progenitors expressing OLIG2, which are rapidly exhausted or converted to oligodendroglial progenitors [26,27]. However, in contrast to mouse motor neurons, which are produced during a brief period between embryonic days 9 and 12, the period of human motor neuron generation spans approximately twenty days [28,29]. This raises the possibility that agents that enhance proliferation of motor neuron progenitors might be used to increase the yield of human motor neurons in culture.

Here we have developed techniques that allow us both to amplify stem cell-derived motor neurons and to perform survival assays in the absence of other cell types. We first report that there is indeed significant ongoing motor neuron generation in cultures of differentiated hESCs. To exploit this so as to increase yield, we therefore screened for compounds that increase the number of motor neurons when applied over this period. We report that the ROCK inhibitor Y-27632 stimulates the proliferation of OLIG2-expressing progenitors, and increases the yield of motor neurons up to four-fold. Using amplified motor neurons from the Hb9::GFP hESC line, we next defined conditions for a robust survival assay using FACS-sorted motor neurons, and used it to demonstrate potent activity for three known neurotrophic factors as well as Y-27632 itself. These approaches should be of general interest for the preparation of human motor neurons on a large scale and for functional and biochemical studies of molecular processes controlling motor neuron genesis, survival and degeneration.

Results

Ongoing motor neuron generation in cultures of differentiated hESCs

To determine whether hESCs differentiated *in vitro* to a mixed spinal cord identity exhibit prolonged motor neurogenesis as in the fetal human spinal cord, we first examined changes in numbers of hESC-derived motor neurons (hESC-MNs) in mixed spinal cultures over a 15-day period using an hESC reporter line that expresses green fluorescent protein (GFP) under the control of the motor neuron-specific murine homeobox gene 9 (Hb9) promoter [23]. We and others previously showed using a range of other markers and functional assays that GFP-positive neurons generated from this line possess many properties of postmitotic motor neurons [6,23,30]. Motor neurons were differentiated from hESCs using a standard protocol involving exposure of embryoid bodies (EBs) to retinoic acid (RA) and recombinant sonic hedgehog protein (SHH) (see *Methods*) [4,6]. After 31 days, EBs were dissociated and cryopreserved to allow multiple experiments to be performed on identical aliquots; however, similar data were obtained using fresh, unfrozen cells (not shown). Cell suspensions were thawed and plated in 96-well plates and automated counts of live motor neurons, defined as GFP^+ neurons with significant neurite outgrowth (SNO, total neurite length >75 μm), were performed (Figures 1A and 1B) [31 33]. In standard culture medium without neurotrophic support motor neuron numbers decreased over the first 7 days, reaching a plateau that was maintained until day 31+13 (Figure 1C and 1D). This did not reflect a loss of reporter expression since a similar decrease was seen when motor neurons were identified by staining for endogenous HB9 (not shown). In contrast, when the medium was supplemented with four neurotrophic factors [NTFs; brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF) and insulin-like growth factor 1 (IGF-1) at 10 ng/mL] in addition to the cAMP-elevating compounds forskolin (F; 10 μM) and iso-butylmethylxanthine (I; 100 μM), after an initial decrease in motor neuron numbers by day 31+7, there was a subsequent increase in the number of hESC-MNs, which reached nearly starting levels by day 31+13 (Figure 1C and 1D).

This late increase in human motor neuron numbers could potentially be explained by ongoing genesis of motor neurons. To assess overall generation of new-born motor neurons we cultured cells with or without NTFs in the continuous presence of the mitotic label 5'-bromo-2'deoxyuridine (BrdU, 2 μM) and counted GFP-positive cells that had incorporated BrdU (Figure 1E). After 15 days, ~60% of all Hb9::GFP cells were positive for BrdU in both conditions (Figures 1F and 1G), but cultures supplemented with NTFs contained 4-fold higher absolute numbers of new-born hESC-MNs (Figure 1G, $p<0.05$). Together, these results demonstrate that human motor neurons are generated over extended periods of culture and that the yield of motor neurons can be increased by treatment with neurotrophic factors.

Screening for small molecules able to increase the yield of human motor neurons

Neurotrophic factors are costly culture supplements and have pleiotropic effects on neural development [16,34,35]. To exploit the high rate of neurogenesis in hESC-MN cultures in a more targeted manner to increase motor neuron yields, we sought to identify available reagents with similar activity. We therefore performed a small-scale screen of 160 bioactive compounds selected from a collection of drug-like chemicals and examined their effect on total motor neuron numbers. We reasoned that the assay might capture two types of compounds, those that increase motor neuron survival and/or others that increase motor neurogenesis. Both types of compounds would be of interest as they could be applied to increase the overall yields of motor neurons derived from hESCs.

Compounds (10 μM in quadruplicate wells) were added on the day of seeding and motor neurons were counted at day 31+13, the time point at which the greatest differences in human motor neuron numbers between control and NTF-supplemented cultures were observed (Figure 1C, $p<0.001$). Most compounds showed no effect, and a significant number resulted in lower motor neuron numbers than the negative control condition (Figure 2A). In contrast, two compounds increased motor neuron numbers by> 1.4 fold compared to basal conditions (Figure 2A). The most significant increase (1.9-fold) was induced by the Rho kinase (ROCK) inhibitor Y-27632 (Figure 2A, Y-27632 vs. No NTFs, $p<0.05$).

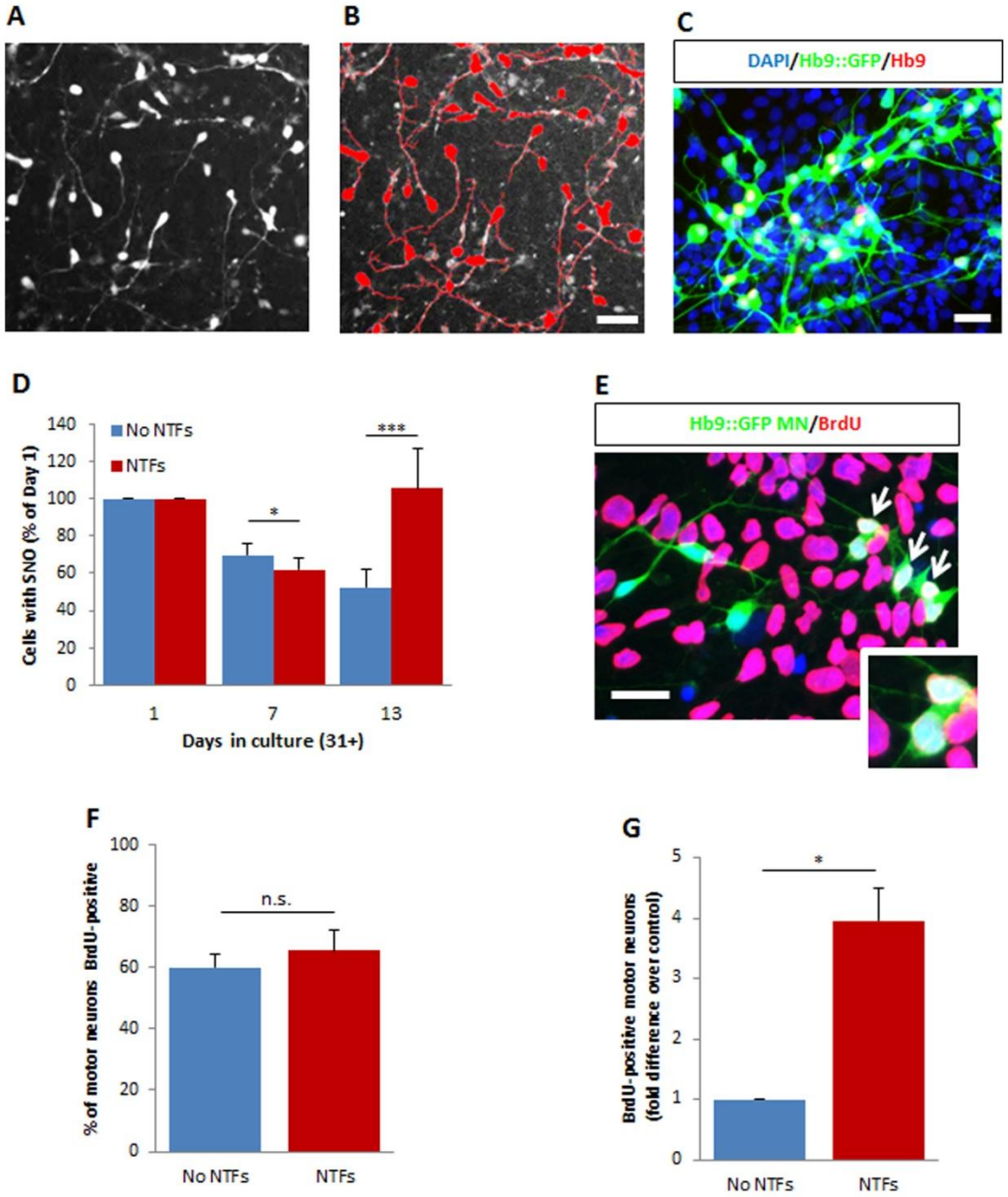

Figure 1. Ongoing birth of motor neurons in hESC-derived cultures is stimulated by neurotrophic factors. (A) Live fluorescent human motor neurons derived from the Hb9::GFP reporter line at day 31+13 after growth with a cocktail of neurotrophic factors (NTFs). (B) Automated quantification of fluorescent cells with significant neurite outgrowth (SNO) using the Neurite Outgrowth module of MetaMorph software; cells counted are identified with a red overlay. Motor neurons were considered to have significant neurite outgrowth when their overall neurite length exceeded 75 μm (scale bar). (C) Representative image of immunostained Hb9::GFP hESC-motor neuron cultures at day 31+13 after growth with a cocktail of neurotrophic factors (NTFs). Scale bar = 50 μM. (D) Number of cells with significant neurite outgrowth (SNO) when grown with (red bars) or without (blue bars) neurotrophic factors, expressed as a percentage of numbers at day 31+1. The increase in motor neuron numbers after day 31+7 in NTF-supplemented cultures suggests ongoing neurogenesis. Surviving fluorescent GFP-positive motor neurons with SNO shown as mean ± s.e.m., $n>5$ (t-test, ***$p<0.001$, *$p<0.05$). (E) BrdU-positive Hb9::GFP-positive motor neurons (arrows) at day 31+15 confirming the presence of newborn human motor neurons in culture. Scale bar = 50 μM. (F) The percentage of Hb9::GFP-positive motor neurons that were BrdU-positive at day 31+15 is not changed by NTFs but (G) total numbers of BrdU-positive motor neurons are increased with NTFs. Bars indicate mean ± s.e.m., $n=3$ (t-test, *$p<0.05$; n.s. = not significant).
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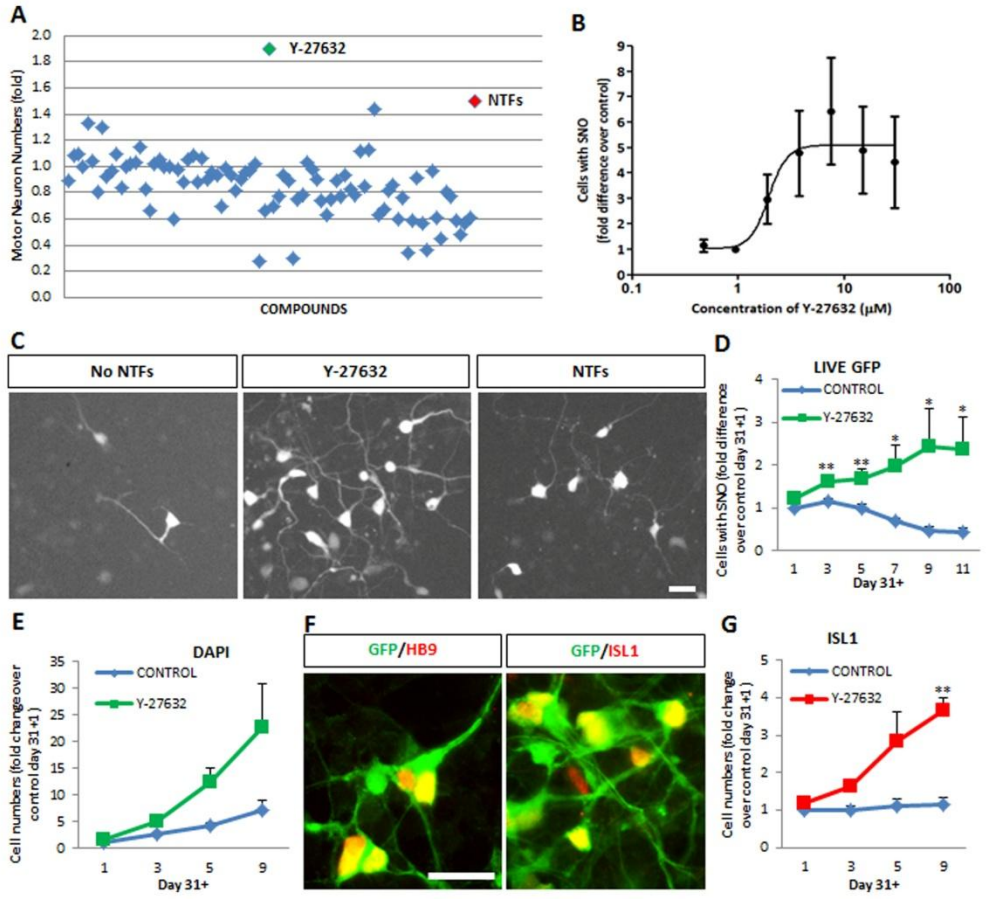

Figure 2. The ROCK inhibitor Y-27632 increases human motor neuron numbers in hESC-derived motor neuron cultures. (A) Screening of 160 compounds for their potential to increase the number of human motor neurons in hESC cultures at day 31+13. Compounds were tested in quadruplicate at a single concentration (10 μM). Values are plotted as mean fold difference in motor neuron numbers relative to the negative control condition (No NTFs). The Rho-kinase (ROCK) inhibitor Y-27632 was the compound showing the highest capacity to increase the number of human motor neurons. (B) Y-27632 increases the number of fluorescent hESC-motor neurons in mixed cultures in a dose-dependent manner. Cells were cultured in the absence of neurotrophic factors and in the presence of increasing concentrations of Y-27632. Values shown as mean ± s.e.m., $n = 4$. (C) Representative images of hESC-motor neuron cultures at day 31+13 grown under neurotrophic factor deprivation (No NTFs), neurotrophic factor supplementation (NTFs + F + I) and Y-27632 (10 μM). Scale bar = 25 μM. (D) Time-dependent increase in the number of motor neurons in the presence (green) but not absence (blue) of Y-27632 (10 μM), with a peak effect at day 31+9. Values shown as mean ± s.e.m., $n > 5$ (t-test, *$p < 0.05$; **$p < 0.01$). (E) Y-27632 also increases the total number of cells in culture. Mean ± s.e.m., $n = 3$. (F) Hb9::GFP-positive neurons continue to express motor neuron markers HB9 and ISL1 after treatment with Y-27632 for 9 days. Scale bar = 50 μM. (G) Supplementation of cultures with Y-27632 (red line) leads to increased numbers of human motor neurons expressing endogenous ISL1 at day 31+9. Mean ± s.e.m., $n = 3$ (**$p < 0.01$).
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The EC_{50} for Y-27632 at day 31+13 was 1.9 μM with a maximum effect of ~5-fold (Figure 2B; $p < 0.05$), even greater than that of neurotrophic factors (Figures 2A and 2C; Y-27632 vs. NTFs + F + I, $p < 0.05$). To optimize the time window for the effects of Y-27632, we next studied the kinetics of human motor neuron generation with or without Y-27632 at its optimal concentration (10 μM). We focused on its effects when added post-dissociation at day 31. Maximum numbers of GFP-positive neurons, representing a ~5-fold increase in motor neuron numbers over basal levels (Figure 2D, $p < 0.05$) were reached at day 31+9, which was therefore adopted as the standard time point for all subsequent experiments.

To exclude the possibility that Y-27632 might have affected the fidelity of HB9 reporter expression, we checked day 31+9 cultures

using direct immunostaining for the motor neuron markers ISL1 and HB9; both showed a high degree of overlap with the GFP reporter (Figure 2F). Moreover, Y-27632 induced a nearly 4-fold increase in absolute numbers of hESC-MNs expressing endogenous ISL1 (Figure 2G, $p<0.05$). Furthermore, to exclude the possibility that the class of motor neurons generated was altered with respect to standard differentiation protocols, we quantified the fraction of GFP-positive neurons expressing FoxP1, a marker for limb-innervating motor neurons, or Lhx3, a marker of medial motor neurons [30,36,37]. Comparable numbers of each class were generated and the ratio was not significantly affected by amplification with Y-27632 ($p>0.05$; not shown). One potential risk of this amplification procedure was that Y-27632 might dilute out motor neurons by stimulating the generation of other cell types. However, this did not appear to negatively affect the outcome: across the many different batches of hES-MNs analyzed in this study, the final abundance of motor neurons ranged from 5% to 45% of total cells, making it important that all treatment groups be compared to controls from the same batch. Although we did not exclude the batches with lower abundance, the value of 45% motor neurons is among the highest reported, demonstrating that expansion did not lead to excessive motor neuron depletion. Thus using a small-scale drug testing approach we were able to identify a compound, Y-27632, which can significantly increase motor neuron numbers in differentiated hESC cultures.

Y-27632 enhances proliferation of motor neuron progenitors in both hESC- and hiPSC-derived cultures

To better understand the level at which Y-27632 exerts its effect, we next examined the expansion of motor neuron progenitors (pMNs), using OLIG2 as a marker [26]. Treatment with Y-27632 increased the number of OLIG2-positive cells ~3.6-fold compared to controls by day 31+9 (Figures 3A and 3B, $p<0.05$) comparable to the ~3.3-fold increase in DAPI-stained cells over controls over the same period [Figure 2E, $p>0.05$; Ratio DAPI/OLIG2 = 6.8:1 (CONTROL) vs. 6.2:1 (Y-27632)]. Application of BrdU from day 31 to day 31+9 led to nuclear labeling of 86% of OLIG2-positive cells, indicating that they are actively proliferating progenitors (Figures 3C and 3D). Accordingly, 74% of GFP-positive motor neurons on day 31+9 were BrdU-positive, indicating that they were born during the period of Y-27632 treatment (Figures 3E and 3F). Similar percentages were observed using fresh, unfrozen motor neuron preparations (not shown). Therefore Y-27632 non-selectively enhances cell proliferation in hESC-derived cultures, resulting in a ~3.5-fold increase in the number of motor neuron progenitors that is likely to contribute significantly to the observed increase in postmitotic hESC-MNs.

To determine whether Y-27632 was a generally effective treatment for pluripotent stem cell lines, we performed similar experiments using an additional hESC line, RUES1; and a hiPSC line, 18c, derived from a healthy control subject [6]. Total numbers of DAPI-stained cells and OLIG2-positive progenitors were quantified as above after 31+9 days. Significant increases in both DAPI-positive and OLIG2-positive cells were observed following Y-27632 treatment using hiPSC 18c (Figures 3G and 3H, $p<0.05$) and for DAPI using RUES1 (Figure 3G). To detect motor neurons in the absence of a reporter we performed immunostaining for HB9, to label motor neurons, and β-III tubulin, to label all neurons (Figures 3I and 3J). Automated image analysis of such cultures revealed a 2- to 4-fold increase in motor neuron numbers (Figure 3I, $p<0.05$). Y-27632 is therefore a useful tool for both hESCs and clinically relevant hiPSC lines.

Design of a robust survival assay for purified human motor neurons

Our overall goal was to study the trophic requirements of human motor neurons. Bulk day 31 cultures were therefore dissociated and grown in the presence of Y-27632 for 3 days or 9 days before FACS analysis, leading to a ~2-fold increase in the total yield of motor neurons after 3 days (Figure 4A; $p<0.01$) and a nearly 4-fold increase after 9 days (Figure 4B; $p<0.01$). For all subsequent experiments, expanded human motor neurons from the day 31+3 time point were used.

Given the ongoing neurogenesis in mixed cultures, it was first necessary to find conditions in which expanded postmitotic neurons could be studied in isolation. Direct treatment of mixed cultures with mitotic inhibitors did not produce satisfactory results: cytosine arabinoside (AraC) proved toxic for human motor neurons, while even the less toxic uridine/fluorodeoxyuridine (U/FdU) led to clumping of neurons on remaining islands of non-neuronal cells (not shown). Motor neurons were therefore FACS-sorted (Figure 4C) and seeded on polyornithine/laminin-coated coverslips in medium containing a cocktail of NTFs plus the c-AMP elevating compounds forskolin and IBMX. Using FACS conditions involving a slow sorting rate and a wide nozzle, the seeded motor neurons rapidly developed robust neurite outgrowth (Figure 4D). To estimate their purity, we performed immunostaining using a combination of antibodies to HB9 and ISL1 ("pan-MN") [30]. At day 31+3+1 (differentiation + expansion + days post-FACS),>95% of the neurons were Hb9::GFP-positive, and reporter expression showed strong overlap with HB9/ISL1 staining (Figure 4D). Despite this high degree of enrichment, colonies of proliferating progenitors were occasionally observed (Figure 4E); sorted motor neurons were therefore cultured in the presence of the antimitotic drug U/FdU (Figure 4E). The new protocol therefore provides a robust and abundant source of highly purified hESC-MNs.

To develop a survival assay based on neurotrophic factor deprivation [31,38,39], FACS-sorted motor neurons were seeded in 96-well plates and stained using the vital dye calcein-AM. This had the advantage that it stained cell bodies and neurites more intensely than live imaging of GFP, which was no longer required to identify motor neurons. Numbers of surviving hESC-MNs were counted in whole culture wells in an automated manner using MetaMorph (Figure 5A). We first asked whether the survival of purified motor neurons was dependent on trophic support in these conditions. At day 31+3+7, motor neuron survival was enhanced ~2.5-fold by a cocktail of NTFs (BDNF, CNTF, GDNF, IGF-1, each at 10 ng/ml) with F (10 μM) plus IBMX (100 μM) (Figure 5B), similar to published results using cultures of primary rodent motor neurons [39 41]. We tested forskolin and IBMX alone and found that they showed only slight innate neurotrophic activity (not shown).

This provided an opportunity to better characterize the effects of known neurotrophic factors on hESC-MNs. Doses of GDNF, BDNF, CNTF and IGF-1 ranging from 2 pg/mL to 10 ng/mL were first tested alone for their effects on survival at day 31+3+7 (Figure 5D to 5G). Except for IGF-1 (not shown), which showed no survival promoting effect alone, each neurotrophic factor provided significant support for human motor neuron survival with EC_{50} values as follows: 2 pM for BDNF, 2 pM for GDNF and 1 pM for CNTF. These are slightly higher than the most potent EC_{50} values reported for the same factors on primary rodent motor neurons (BDNF, EC_{50} = 1 pM [38]; GDNF, EC_{50} = 0.2 pM [31]; CNTF, EC_{50} = 0.1 pM [18,42]); this may reflect differences related to species, human stem cell origin or batch of neurotrophic factor. Since the effects of neurotrophic factors on rat motor

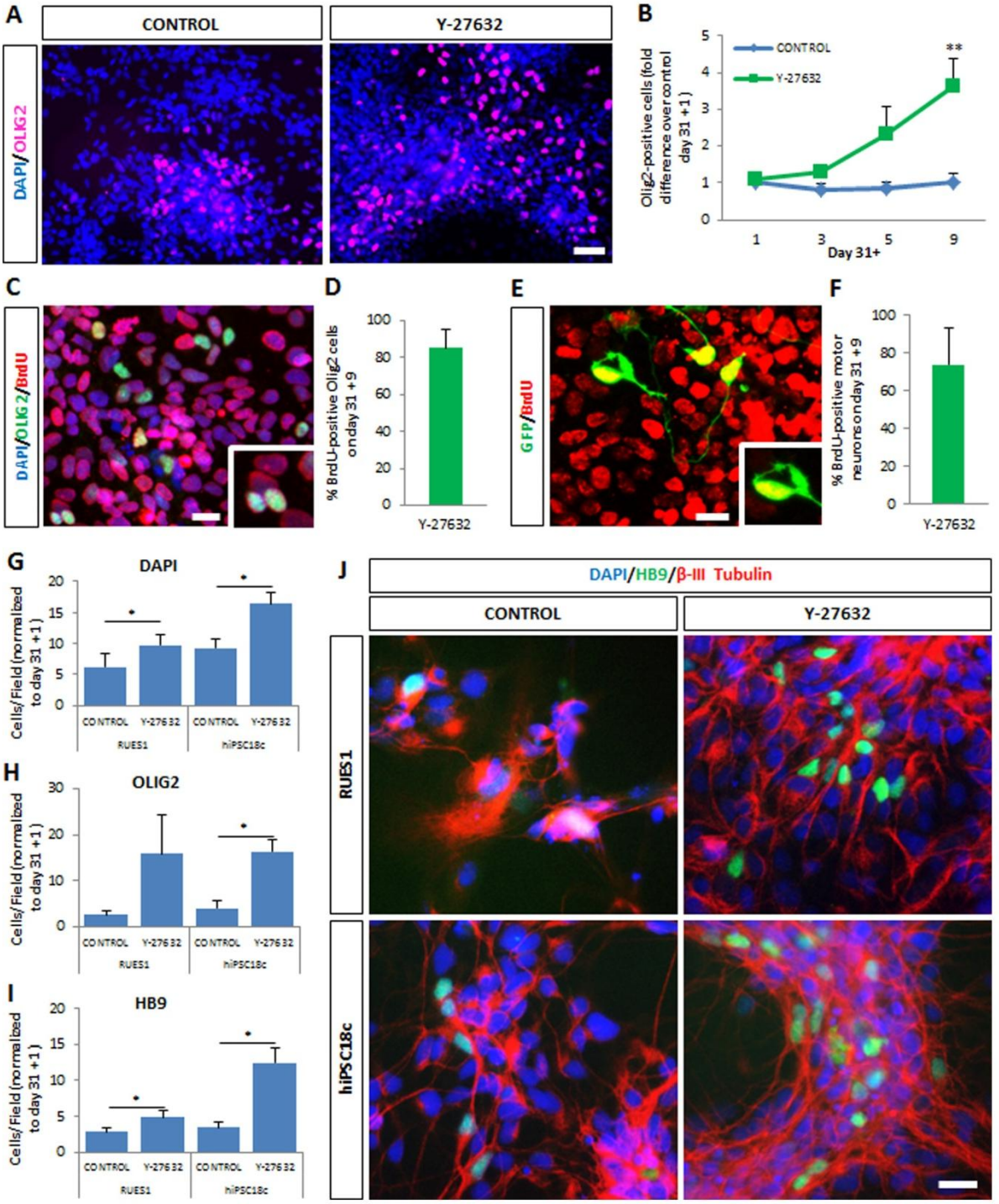

Figure 3. Y-27632 enhances proliferation of motor neuron progenitors in hESC- and hiPSC-derived motor neuron cultures. (A) Y-27632-supplemented cultures contain increased numbers of OLIG2-positive cells at day 31+9. Scale bar = 50 μM. (B) Time-dependent increase in numbers of OLIG2-expressing progenitors in the presence of Y-27632. Data normalized to control at day 31+1; mean ± s.e.m., $n>5$ (t-test, **$p<0.01$). (C) OLIG2 progenitors at day 31+9 stained for BrdU. Scale bar = 25 μM. (D) Percent of OLIG2 precursors that are BrdU-positive at day 31+9 (mean ± s.e.m., $n=4$). (E) Hb9::GFP-expressing motor neurons at day 31+9 stained for BrdU. Scale bar = 25 μM. (F) Percent motor neurons that are BrdU-positive at day 31+9 (mean ± s.e.m., $n=4$). (G) The total number of cells in culture is increased at day 31+9 following Y-27632 treatment of hESC

RUES1 and hiPSC18c. Values are mean ± s.e.m., $n \geq 3$ (t-test, *$p<0.05$). (H) Numbers of OLIG2 precursors increase significantly at day 31+9 following Y-27632 treatment of hiPSC 18c. Values are mean ± s.e.m., $n \geq 3$ (t-test, *$p<0.05$). (I) Numbers of motor neurons identified by staining for endogenous HB9 increase significantly at day 31+9 following Y-27632 treatment of hESC RUES1 and hiPSC 18c. Values are mean ± s.e.m., $n \geq 3$ (t-test, *$p<0.05$). (J) Cultures from healthy control hESCs (RUES1) or hiPSCs (18c) immunostained for the motor neuron marker HB9 and the pan-neuronal marker β-III tubulin. Y-27632 increases the number of motor neurons in each case. Scale bar = 25 μM.
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neurons in defined media was reported to depend on intracellular cAMP levels [40,41], we also tested the effects of inclusion of forskolin and IBMX (F+I). The neurotrophic activity of each factor tested appeared to be increased in the presence of F+I, though this effect was only significant for single points at the highest concentration of GDNF (Figure 5D-F). To determine whether different neurotrophic factors were potentially acting on different subsets of motor neurons in the cultures, we next performed a dose-response analysis for a combination of all factors with a fixed concentration of F+I (Figure 5H). The maximum number of motor neurons maintained in culture was not significantly greater than that with BDNF, CNTF or GDNF alone (with F+I). This suggests that essentially all viable motor neurons are maintained by optimal doses of these single factors, at least after 7 days in culture. Therefore, like their rodent counterparts, human motor neurons show an exquisitely sensitive response to multiple neurotrophic factors.

Lastly, to evaluate the ability of the newly developed human motor neuron survival assay to detect novel neurotrophic compounds, we determined whether the beneficial effect of Y-27632 on human motor neuron numbers, in addition to its effect on progenitor proliferation, might also reflect a survival effect. To exclude effects on cell attachment we first verified that the presence of the drug did not affect hESC-MN numbers after 24 hours (Figure 6A). After 7 days in culture, Y-27632 had a clear dose-dependent survival effect (Figures 6B and 6C), though to a more modest extent than neurotrophic factors. The EC_{50} for the Y-27632 survival effect on motor neurons was 2 μM, similar to the value for motor neuron expansion. Thus Y-27632 not only promotes proliferation of motor neuron progenitors but also functions as a motor neuron survival factor. The fact that the fold-increase in survival was lower than that induced in long-term treatment of mixed cultures (Fig. 2B), likely reflects the absence of proliferation and/or other cell types.

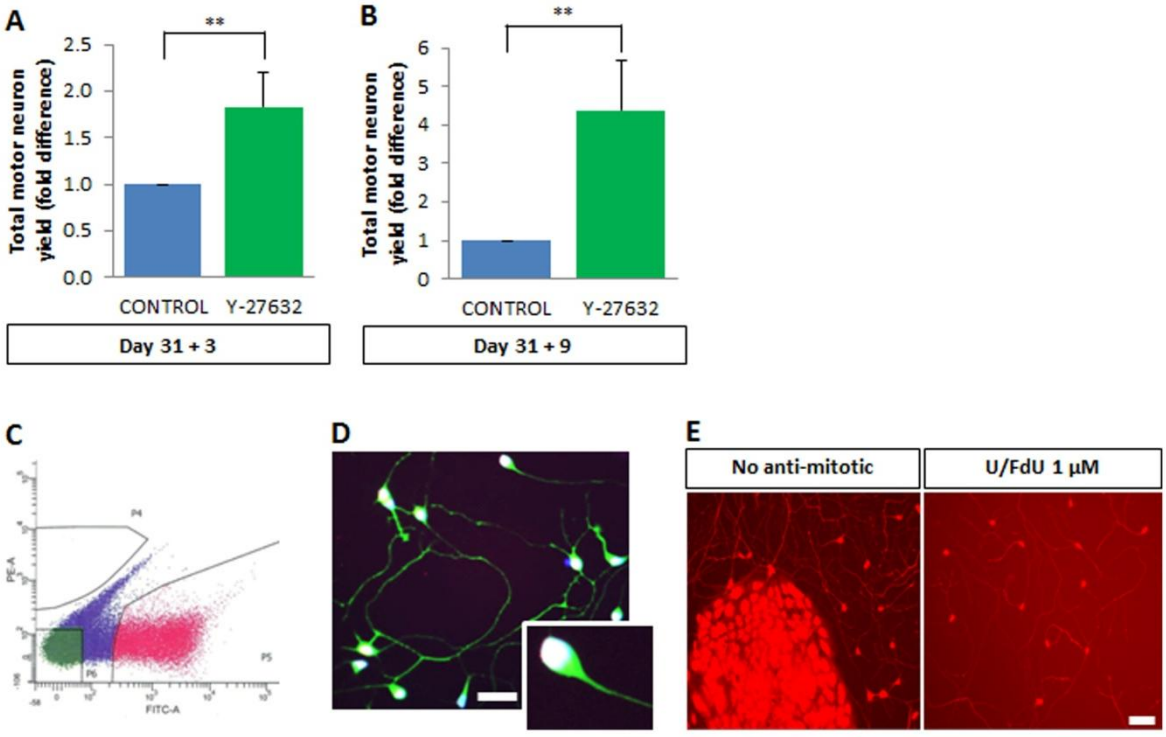

Figure 4. FACS-sorting of amplified cultures yields a pure preparation of viable human motor neurons. (A) Y-27632 supplementation for 3 days leads to a 1.8-fold increase in motor neuron yield judged by FACS analysis. Data normalized to controls without Y-27632. Values are mean ± s.e.m., $n>5$ (t-test, **$p<0.01$). (B) Nine-day treatment with Y-27632 gives a ~5-fold increase in motor neuron yield as compared to controls without Y-27632, as quantified by flow cytometry. Values are mean ± s.e.m., $n>5$ (t-test, **$p<0.01$). (C) FACS purification of Hb9::GFP motor neurons expanded with Y-27632 for 3 days. Representative FACS gating used to retrieve an almost pure (>95%) population of human motor neurons. (D) FACS-purified motor neurons at day 31+3+1 stained for GFP (green), and a combination of HB9 and ISL1 ("pan-MN"; white nuclei).>95% of the FACS-purified cells in culture are Hb9::GFP positive. Scale bar = 25 μM. (E) Even following FACS sorting, some contaminant cells were able to proliferate and form colonies that interfered with survival assays (left panel). Uridine/Fluorodeoxyuridine (U/FdU) (each at 1 μM) successfully prevented the proliferation (right panel).
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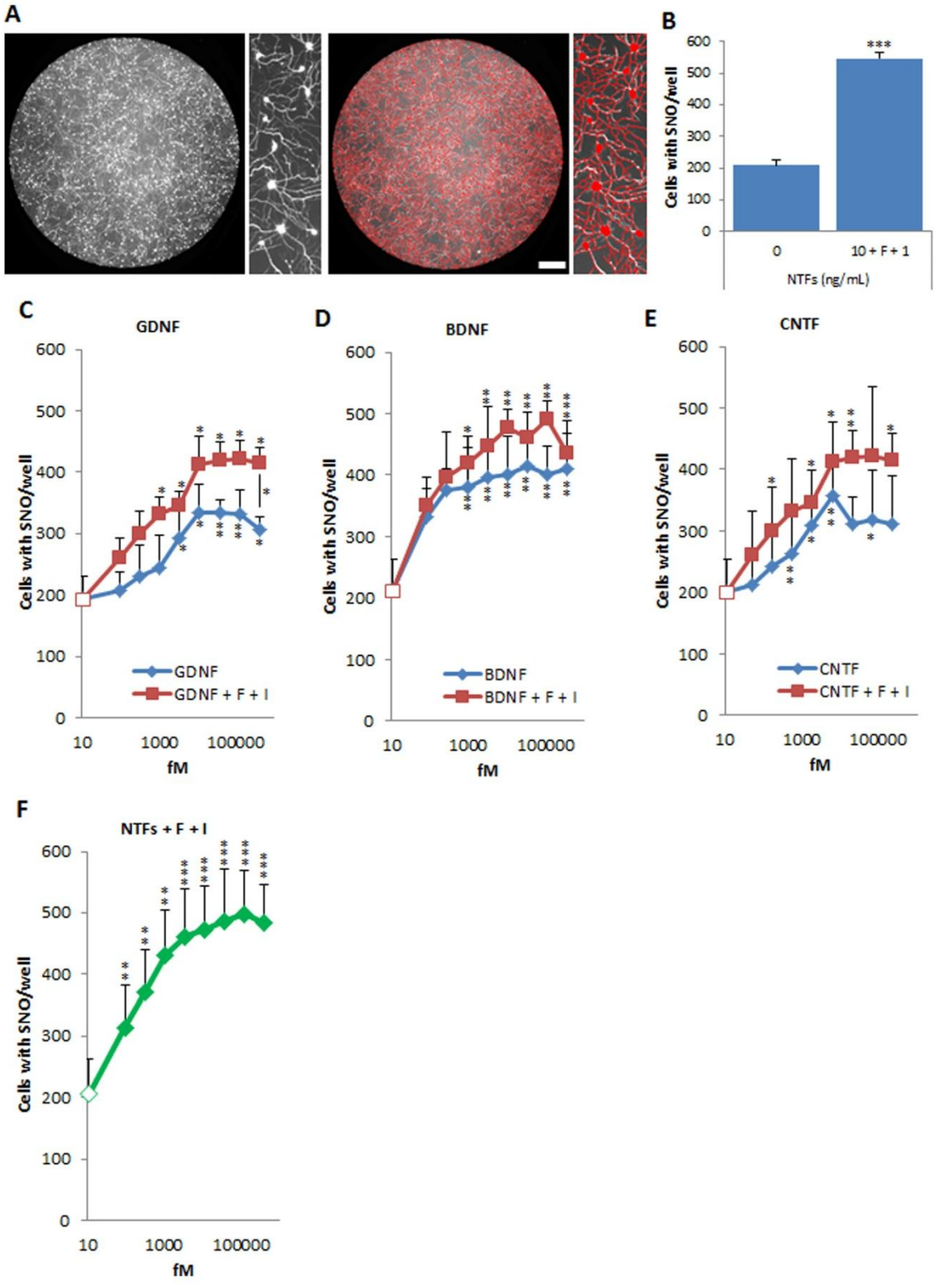
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Figure 5. Purified human motor neurons show a potent response to known neurotrophic factors. (A) Whole-well imaging of live motor neurons labeled with calcein-AM captured using the Plate Runner (left two panels). Surviving human motor neurons were counted in whole culture wells in an automated manner using MetaMorph (red tracing, right two panels). Scale bar = 200 μm. (B) Y-27632-expanded motor neurons show enhanced survival in the presence of a cocktail of neurotrophic factors. Values shown as mean ± s.e.m., n>5 (t-test, ***p<0.001). (C) GDNF, (D) BDNF and (E) CNTF alone (blue lines) enhance the survival of expanded FACS-purified human motor neurons. The addition of F+I significantly potentiates the survival-inducing activity of GDNF at high concentrations. Values shown as mean ± s.e.m., n>4 (t-test, *p<0.05; **p<0.01; ***p<0.001). Asterisks on individual points represent significance of difference with No-NTF control (white rectangle in the curve); asterisks on bars represent significant differences between a given concentration of NTF and the corresponding value for NTF + F+ I. (F) The cocktail of neurotrophic factors (NTFs) enhances the survival of expanded FACS-purified human motor neurons in a dose-dependent manner in the presence of 10 μM forskolin plus 100 μM IBMX. Values shown as mean ± s.e.m., n≥5 (t-test, **p<0.01; ***p<0.001).
doi:10.1371/journal.pone.0110324.g005

Discussion

Human embryonic and induced pluripotent stem cells (hESCs and hiPSCs) represent a powerful tool for studying human development, disease modeling and drug discovery. However, one major limiting factor for prospective drug screens is the efficiency with which the affected cell types can be generated and, in the case of motor neurons and many other neuronal classes, the absence of a validated survival assay. Here, we took advantage of our observation of ongoing motor neuron generation in hESC-derived cultures to devise a new method for amplification of motor neuron progenitors to increase motor neuron yields. In addition, using optimized conditions for FACS sorting of neurons expressing the Hb9::GFP reporter, we developed a robust assay for survival factors acting directly on postmitotic motor neurons, and used it to show that human motor neurons respond in a potent manner to both known and novel neurotrophic molecules.

The ongoing neurogenesis in human motor neuron cultures that we describe contrasts with the short ~24-hour period of motor neuron production in differentiated mouse ES cell cultures [43]. This is likely to reflect normal biological differences in the development of motor systems in rodent and human embryos, since human motor neurons are produced over an extended three-week period *in vivo* [28,29]. We first exploited this to screen for compounds that would further amplify the precursor population, identifying Y-27632 as the most active compound in a screen which, like higher-throughput assays, was carried out at a single concentration. Exactly how Y-27632 is achieving this may involve multiple mechanisms, but we considered three potential modes of action for Y-27632 in increasing numbers of motor neuron progenitors. First, it could act by blocking differentiation of progenitors to motor neurons. This seems unlikely since the numbers of OLIG2-positive precursors and motor neurons increased in parallel. Second, it might specifically promote the generation of OLIG2-positive precursors. Since total DAPI numbers increased in parallel, such a selective effect seems unlikely. We therefore believe Y-27632 acts by shortening cell cycle time for dividing precursors as a whole, leading to expansion – but not enrichment – of motor neuron progenitors and a subsequent increase in motor neuron yield [44–46].

Nevertheless, the ongoing neurogenesis also provides a potentially serious confound for interpretation of experiments examining changes in motor neuron numbers in mixed cultures. In studies that do not take this into account, it is possible that an increase of motor neuron numbers attributed to improved survival may instead reflect an effect on neurogenesis. To overcome this issue, we FACS-purified motor neurons derived from the Hb9::GFP hESC line and cultured them alone in the presence of a mitotic inhibitor U/FdU to inhibit proliferation of any remaining progenitors. This is in some ways analogous to the approach recently reported by Yang et al. [24], except that to block proliferation they used cytosine arabinoside, which was cytotoxic in our hands. Moreover, their cell survival experiments, performed over a 20-day period, required a mouse astrocyte monolayer as substrate, whereas our cultures contained essentially only motor neurons.

Using this essentially pure preparation of postmitotic motor neurons we showed that three known neurotrophic factors potently enhance human motor neuron survival, and that their action is potentiated when endogenous levels of cAMP are

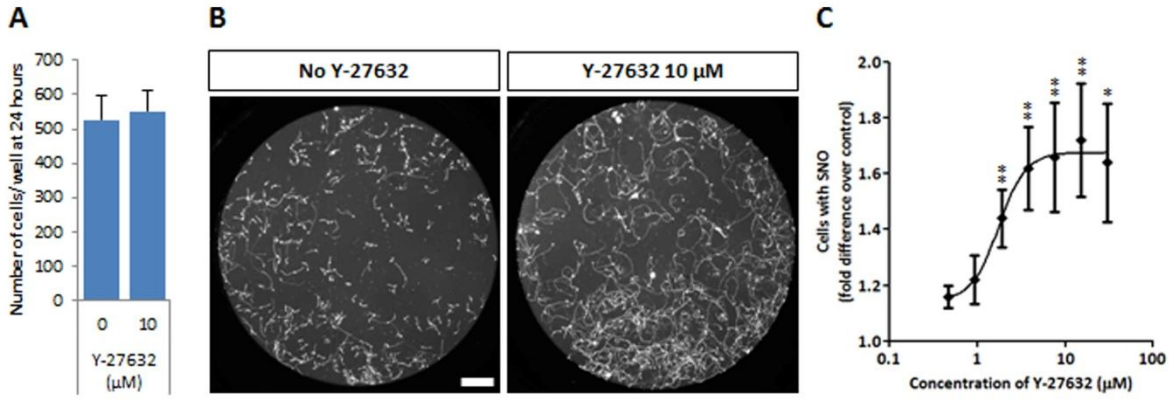

Figure 6. Y-27632 is also a survival factor for human motor neurons. (A) The plating efficiency of FACS-purified human motor neurons after 24 hours is not increased in the presence of Y-27632. (B) Y-27632 enhances the survival of FACS-purified human motor neurons in a 7-day survival assay. Scale bar = 200 μM. (C) Dose-dependent effects of Y-27632 on human motor neuron survival, expressed relative to the basal condition (0 μM). Values shown as mean ± s.e.m., n≥5 (t-test, *p<0.05; **p<0.01).
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increased. Therefore, in this respect, the human stem cell-derived neurons closely resemble rodent motor neurons both in primary culture and *in vivo*. Since dependence on trophic factors is acquired over time during embryogenesis [47], this also suggests that the human motor neurons have reached a stage of maturation comparable to those in the mid-embryonic period in mice.

Y-27632 has been shown to have contrasting biological effects in different systems, ranging from pro-proliferative effects on hESCs and hiPSCs [48,49] to anti-proliferative effects on cancer cells [50], cord blood-derived $CD34^+$ hematopoietic progenitor cells [51], hepatic stellate cells [52] and smooth muscle cells [53]. While it is neuroprotective for primary mouse Purkinje cells [54], retinal ganglion cells [55] and murine hippocampal slice cultures [56] and growth-promoting for corticospinal tract axons [57,58] and adult optic nerve [59], Y-27632 is not protective for hiPSC-derived dopaminergic neurons [60]. Our study extends others which suggest that Y-27632 exhibits generally beneficial effects on motor neurons. A recent report documented an increase in the lifespan of an intermediate mouse model of SMA following administration of fasudil, another ROCK inhibitor [61]. Even though the compound was not able to halt motor neuron loss in the ventral horn of the spinal cord, positive effects on the maturation of the neuromuscular junction and muscle fiber size were reported [61]. More recently, fasudil was reported to extend survival and reduce motor neuron death - in a mouse model of amyotrophic lateral sclerosis [62]. Therefore, the neurotrophic properties of Y-27632 described here for cultured human neurons likely reflect a mechanism of action that is conserved across species and *in vivo*.

In summary, our study defines conditions for systematic assays of neurotrophic factors and survival-promoting compounds for human motor neurons. We show that the technique can be extended to human iPSC-derived motor neurons and therefore in principle to comparisons between cells derived from ALS patients and controls: we and others recently derived Hb9::GFP or Hb9::RFP reporters for different ALS-iPSC lines [63]. Importantly, in agreement with our earlier studies on the expression of specific markers, electrophysiological characteristics and development following transplantation [6,30], we show that the neurotrophic dependence of human stem cell-derived motor neurons has reached a state of maturity comparable to that of primary embryonic motor neurons *in vitro* and *in vivo*. Although more still needs to be done before they can be considered to reflect the properties of the postnatal spinal cord, this validates their use as a human model for analyzing multiple aspects of motor neuron development and pathology.

Materials and Methods

Cell lines

All the human ES and iPSC lines have been reported in an earlier publication [6]. The iPS cell lines were derived by retroviral transduction of OCT4, SOX2, and KLF4 in dermal fibroblasts. All pluripotent cell lines were characterized by conventional methods and grown under standardized conditions as described below.

Ethics Statement

The work performed with human motor neurons derived from hESCs and hiPSCs has been approved by Columbia University ESCRO committee (Embryonic Stem Cell Research Oversight committee). Patient fibroblasts for generating human iPS lines were collected with written informed consent under IRB approval AAAC1257 from Columbia University Medical Center.

Growth of hPSC lines

We used an HB9::GFP reporter hESC line [23], the wild-type hESCs line RUES1 and hiPSC line 18c [6]. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Human ESCs and hiPSCs were grown on a pre-gelatinized tissue culture flask on a monolayer of irradiated CF-1 mouse embryonic fibroblasts (MEFs; GlobalStem) plated at 15,000 18,000 cells/cm^2 in hPSC medium [DMEM/F12 (Invitrogen), 20% knockout serum replacement (Invitrogen), 1 mM L-glutamine (Gibco), 100 μM non-essential aminoacids (Gibco) and 100 μM β-mercaptoethanol (Sigma-Aldrich)] supplemented with 20 ng/ml recombinant human basic fibroblast growth factor (bFGF; R&D Systems). Medium was changed every day for the duration of the expansion and lines were passaged every 4 6 days using dispase (Gibco) at 1 mg/mL in hPSC medium for 30 minutes at 37°C.

Differentiation of hESCs and hiPSCs into motor neurons

hESCs and hiPSCs were allowed to reach 75% 90% confluency. Then, colonies were treated with dispase (1 mg/mL) to separate colonies from the MEF layer. After 30 minutes, cells were washed off the flask using hPSC medium and collected in a 50 mL Falcon tube. Colonies were allowed to settle by gravity and then medium was aspirated. Fresh hPSC medium was added to the cells. This step was repeated three times to wash away all the remaining dispase. Settled colonies were then mechanically dissociated into small 10- to 15-cell chunks using a P1000 tip by performing up and down movements in a 1 mL volume. Cell aggregates were transferred to low adherence T75 flasks in hPSC medium with 20 ng/mL bFGF and 20 μM Y-27632 (Ascent) for the first 24 hours. At day 1, cells for all experiments were supplemented with hPSC medium containing 20 ng/mL bFGF, 20 μM Y-27632, 10 μM SB431542 (Sigma-Aldrich) and 0.2 μM LDN193189 (Stemgent). The medium was changed daily from day 2 to day 4. At day 5, embryoid bodies (EBs) were switched to medium composed of 50% hPSC medium and 50% neural induction medium [NIM; DMEM/F12 (Invitrogen), 1% N2 supplement (Invitrogen), 1 mM L-glutamine (Gibco), 100 μM non-essential aminoacids (Gibco) and 2 μg/mL heparin (Sigma-Aldrich)] supplemented with 10 μM SB431542, 0.2 μM LDN193189, 10 ng/mL recombinant human brain-derived neurotrophic factor (BDNF; R & D Systems), 0.4 μg/mL ascorbic acid (Sigma-Aldrich) and 1 μM retinoic acid (Sigma-Aldrich). At Day 7 cells were switched to 100% NIM, keeping the same medium supplementation. Every other day between days 9 and 21, NIM supplemented with 10 ng/mL BDNF, 0.4 μg/mL ascorbic acid, 1 μM retinoic acid and 200 ng/mL recombinant C25II modified sonic hedgehog protein (SHH; Invitrogen) was added to the EBs. At day 22, cells were cultured with 50% NIM and 50% neural differentiation medium [NDM; Neurobasal (Invitrogen), 1% N2 Supplement (Invitrogen), 1 mM L-Glutamine (Gibco) and 100 μM Non-Essential Aminoacids (Gibco)] supplemented with 2% B-27 supplement (Invitrogen), 0.4 μg/mL ascorbic acid, 1 μM retinoic acid, 200 ng/mL SHH (Invitrogen), 10 ng/mL BDNF, 10 ng/mL recombinant human ciliary neurotrophic factor (CNTF; R & D Systems), 10 ng/mL recombinant human glial cell line-derived neurotrophic factor (GDNF; R & D Systems) and 10 ng/mL recombinant human insulin-like growth factor 1 (IGF-1; R & D Systems). Between days 24 and 31, the bulk medium was switched to 100% NDM and the EBs grown under the previous medium supplementation. After 31 days of differentiation the EBs were dissociated and the resulting neuronal cultures cryopreserved. Briefly, the EBs were collected in a 50 mL Falcon tube and then washed twice with PBS without Ca^{2+} and Mg^{2+} (Invitrogen) to eliminate residual media. The EBs were then incubated at 37°C

in pre-warmed 0.05% Trypsin-EDTA (Invitrogen) for 5 10 minutes. Lastly, fetal bovine serum (Invitrogen) supplemented with 100 μg/mL deoxyribonuclease I (DNAse I, Sigma-Aldrich) was added to stop the trypsin reaction and the cells were spun for 5 minutes at 400×*g*. The cells were resuspended in 1 mL of complete trituration and wash medium [CTWM, PBS without Ca^{2+} and Mg^{2+}, 25 mM Glucose (Sigma-Aldrich), 4% L-15 dialyzed BSA (Sigma-Aldrich), 100 μg/mL DNAse I, 1% N2 supplement, 2% B27 supplement, 600 mM magnesium chloride (Sigma-Aldrich), 500 nM EDTA (Sigma-Aldrich) and 2% FBS] and subsequently mechanically triturated using a P1000 tip. The resulting cell suspension was filtered using a 40 μM cell strainer (BD Falcon) to eliminate large residual clumps and centrifuged for 5 minutes at 400×*g*. The cells were then resuspended in NDM supplemented with 2% B27, 0.4 μg/mL ascorbic acid, 25 μM glutamate E (Sigma-Aldrich), 25 μM β-mercaptoethanol (Millipore), 0.1 μM retinoic acid, 10 ng/mL BDNF, 10 ng/mL CNTF, 10 ng/mL GDNF and 10 ng/mL IGF-1. These cells were counted and then prepared for cryopreservation using 2x Freezing Media (Millipore). Vials of 5 10 million cells/mL were prepared to be used in further experiments.

Coating of 96-well plates

All survival and proliferation studies were performed in 96-well plates (Greiner Bio-One) coated with polyornithine (Sigma-Aldrich) and mouse laminin (Invitrogen). Briefly, 100 μg/mL polyornithine (Sigma-Aldrich) in cell culture water was added to the wells for at least 2 hours then aspirated and the wells rinsed once using water. Coating was completed by adding overnight 15 μg/mL mouse laminin in L15 medium (Sigma-Aldrich) supplemented with 7.5% sodium bicarbonate (Gibco). In studies involving FACS-sorted cells, a concentration of 1000 μg/mL polyornithine was used for coating.

Studies involving mixed hPSC-derived motor neuron cultures

All proliferation/survival studies involving mixed hPSC-derived motor neuron cultures were started from previously cryopreserved vials. After quickly thawing the vials in a 37°C water bath, cells were resuspended in NDM medium supplemented with 2% B27 Supplement. Cells were then spun at 400×*g* for 5 minutes. The supernatant was gently aspirated and cells resuspended in 10 mL of NDM with 2% B27. A 4% BSA protein cushion was then layered under the cell suspension and the cells spun at 400×*g* for 5 minutes, with low acceleration and deceleration. Afterwards, cells were resuspended in basal medium (BM) [Custom Clear Neurobasal (Invitrogen), which omits phenol red and riboflavin to allow live fluorescent imaging in the presence of a significantly attenuated auto-fluorescent background; 1 mM L-glutamine and 100 μM non-essential aminoacids, 2% B27, 0.4 μg/mL ascorbic acid, 25 μM glutamate E, 25 μM β-mercaptoethanol, 0.1 μM retinoic acid] and counted using a hemocytometer. Finally, cells were resuspended at the final desired seeding concentration of 32,000 cells/well and 100 μL was added to each well. Cells were allowed to attach at 37°C for 2 hours before addition of supplements at 3x concentration in 50 μL of BM.

Screening for small molecules with the potential to increase numbers of human motor neurons in culture

From a collection of drug-like chemicals from the Microsource and Tocris collections, two plates containing a total of 160 compounds were selected. Each compound was tested at 10 μM. Basal medium to dilute compounds from original stocks was M-199 (without phenol red; Invitrogen) with 5% DMSO (100% anhydrous, Fisher Scientific), freshly prepared. Survival in BM was used as negative control (trophic factor deprivation). Survival in BM supplemented with a cocktail of NTFs [BDNF, CNTF, GDNF and IGF-1] plus the cAMP-elevating compounds forskolin (F; 10 μM; Sigma-Aldrich) and isobutylmethylxanthine (I; 100 μM; Sigma-Aldrich) was the positive control (trophic factor supplementation). Cells were seeded at 32,000 cells/well in 150 μL and compounds added in a 15 μL volume (the final DMSO concentration of 0.45% did not adversely affect motor neuron survival when added alone, not shown). The same volume of M-199 with 5% DMSO was added to the negative control wells. NTFs + F + I were also added in 15 μL of M-199 with 5% DMSO in positive control wells. For each set, compounds were tested in quadruplicate by creation of 4 test plates. In each plate, each control condition (positive and negative) had six replicate wells. Readouts were performed on day 31+13. After quantification of the total number of surviving cells with significant neurite outgrowth (see *Results*), data were plotted as mean fold difference as compared to numbers in the negative control condition. Plates were rejected when the mean difference in cells numbers between positive and negative control was lower than 1.3 fold. Validation of the most active compounds was performed by serial dose response studies.

Immunocytochemistry

Neuronal cultures were pre-fixed by adding one volume of 4% paraformaldehyde diluted in phosphate-buffered saline 1x (PBS1x/4%PFA) for 2 minutes at room temperature. Then, cells were fixed with PBS1x/4%PFA for 30 minutes at 4°C. After fixation, cells were washed with PBS1x three times for 5 minutes and then permeabilized and quenched for at least 30 minutes using PBS1x with 0.1% Triton-X (PBSTX-0.1%) supplemented with 100 mM glycine and 0.1% Sodium Azide (Sigma-Aldrich). Cells were blocked in PBSTX-0.1% containing 10% donkey serum (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich) (blocking solution) for one hour. After blocking, cells were incubated overnight at 4°C with primary antibodies diluted in the blocking solution. Primary antibodies used in this study were the following: rabbit anti-GFP (1:3000, Abcam), mouse anti-ISL1 (1:200, DSHB, 39.4D5), guinea-pig anti-ISL1 (1:2000, courtesy of Susan Brenner-Morton, Jessell laboratory at Columbia University), mouse anti-HB9 (1:100, DSHB, MNR2 81.5C10-c), chicken anti-β-III Tubulin (TUJ1, 1:1000, Neuromics), rabbit anti-Olig2 (1:1000, Millipore) and rat anti-BrdU (1:150, Serotec). Cells were washed five times with PBSTX-0.1% for 5 minutes. Antigens were visualized by incubating for 60 75 minutes at room temperature with the appropriate secondary antibodies (DyLight 488, 549 and 649 conjugated, 1:1000, Jackson ImmunoResearch). Lastly, neuronal cultures were again washed five times with PBSTX-0.1% for 5 minutes and incubated in a solution containing DAPI (1:50000, Sigma-Aldrich) for 15 minutes. Cells were washed once with PBSTX-0.1% and then imaged.

BrdU incorporation studies

5-bromo-2-deoxuridine (BrdU) incorporation studies were performed to analyze cell proliferation. Neuronal cultures were incubated with BrdU (2 μM; Sigma-Aldrich) for the full duration of culture until fixation with PFA. The standard protocol for immunochemistry described above was followed for other antigens besides BrdU. Then, to detect BrdU incorporation, cells were again pre-fixed for 2 minutes at room temperature and fixed with PBS1x/4% PFA for 15 minutes at 4°C. They were then washed with PBS1x three times for 5 minutes. Cells were then incubated

with pre-warmed (37°C) 2 M HCl in distilled water for 10 minutes at 37°C, light protected. Lastly, the HCl was aspirated and cells were incubated in 0.15 M boric acid in distilled water for 2 minutes at room temperature. Cells were then washed three times with PBS1x for 5 minutes and blocked for 1 hour using blocking solution. Finally, cells were incubated overnight at 4°C with rat anti-BrdU primary antibody (1:150, Serotec) in blocking solution. In order to correct for any non-specific background staining, the same procedures were performed on samples incubated or not with BrdU.

FACS purification and motor neuron survival studies

HB9::GFP reporter hESC-derived motor neurons were grown in polyornithine/laminin-coated T75 flasks prior to FACS purification in order to maximize the amount of cells retrieved after the procedure. After the expansion period, the medium was aspirated and cells washed once with PBS without Ca^{2+} and Mg^{2+} to eliminate residual medium. The cells were then incubated at 37°C in pre-warmed 0.05% Trypsin-EDTA for 5 minutes. DNAse I-supplemented FBS was used to stop the trypsin reaction. Cells were collected and centrifuged for 5 minutes at 400×*g*. Cells were resuspended in complete trituration and wash medium [CTWM, PBS without Ca^{2+} and Mg^{2+}, 25 mM Glucose (Sigma-Aldrich), 4% L-15 dialyzed BSA (Sigma-Aldrich), 100 µg/mL DNAse I, 1% N2 supplement, 2% B27 supplement, 600 mM magnesium chloride (Sigma-Aldrich), 500 nM EDTA (Sigma-Aldrich) and 2% FBS], filtered through a 40 µM Cell Strainer (BD Falcon) and centrifuged for 5 minutes, at 400×*g*. The cells were then resuspended in 750 800 µL of CTWM and transferred to a sorting tube (BD Falcon). Cells were sorted based on GFP expression using a BD FACS Aria II sorter (Becton Dickinson) configured with a 100 µm ceramic nozzle and operating at 20 psi for no longer than 30 minutes. Purified cells were collected in a tube containing CTWM. After collection cells were spun for 5 minutes at 400*xg* and resuspended in Basal FACS Medium [Basal Medium with Clear Custom Neurobasal, supplemented with 1 µM uridine/fluorodeoxyuridine (U/FdU; Sigma-Aldrich), 100 Units/mL Penicillin (Invitrogen), 100 µg/mL Streptomycin (Invitrogen) and 100 µg/mL Normocyn (InvivoGen)]. After cells were counted using a hemocytometer, they were resuspended at the final seeding concentration of 2000 cells/well and added to the wells in 100 µL. Medium supplements were added to the cells at 3x concentration in 50 µL of Basal FACS Medium after the cells were allowed to incubate at 37°C for 2 hours in order to attach to the bottom of the plate. Readouts were performed after 7 days.

Calcein live imaging

To facilitate the imaging of FACS-sorted GFP-positive cells, we used the Calcein-AM Red-Orange (Invitrogen) vital dye at 2.5 µM concentration. Briefly, cells were incubated with the dye for 5 minutes and then extraneous fluorescence was quenched using 5 mg/mL hemoglobin before image acquisition using the Plate Runner (Trophos).

Image acquisition and quantitative image analysis

Image acquisition was performed using either a Carl Zeiss Observer Z1 epi-fluorescence Microscope (Carl Zeiss Inc.; acquisition of 12 images per well at 10x magnification) or the whole well imaging device Plate Runner (Trophos). Automated quantitative image analysis of fluorescent surviving hESC-MNs and stained neuronal cultures was performed using the MetaMorph Software V7.6 (Molecular Devices). The Neurite Outgrowth application in the software was employed to quantify fluorescent human motor neurons that have neurite outgrowth above a certain threshold, reducing the number of false-positive cells such as non-viable neurons included in the analysis. Quantitative analysis of stained hPSC-derived human motor neuron cultures was performed using the Multi-Wavelength Cell Scoring application. For a specific marker, positive cells were selectively identified as having clear signal intensity above local background. Intensity thresholds were set blinded to sample identity. In a given experiment the same parameters were used in all images analyzed. Parameters were only minimally adjusted across different experiments.

Statistical Analyses

All quantitative data were analyzed using IBM SPSS Statistics 19 (IBM SPSS). For each set of data a double statistical evaluation was performed: A) for each condition/time point mean values were compared using one-way ANOVA statistical evaluation followed by Tukey HSD Post-hoc test; B) possible interactions between time and condition were assessed using two-way ANOVA statistical evaluation. In cases involving only one time point and a two-group comparison, p value was determined using Student's t-test. Differences were considered to be significant when $p<0.05$.

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Author Contributions

Conceived and designed the experiments: NJL BJK LR HW CEH. Performed the experiments: NJL BJK YK AGD. Analyzed the data: NJL BJK LR YK AGD HW CEH. Wrote the paper: NJL HW CEH.

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CHAPTER 3.2

Nuno Jorge Lamas, Hynek Wichterle and Christopher E. Henderson

Y-27632 supplementation during hESC-derived motor neuron differentiation fails to increase motor neuron yields

(*Abstract*)

2014

Y-27632 supplementation during hESC-derived motor neuron differentiation fails to increase motor neuron yields

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ABSTRACT

The ability to efficiently generate large quantities of human motor neurons from human embryonic stem cells (hESCs) under defined conditions opens the prospect of conducting relevant studies using standard batches of those treasurable neuronal cells. Current protocols for motor neuron differentiation from hESC are lengthy, costly and result in the generation of mixed populations of neurons of interest and other non-neuronal cells. We have previously demonstrated that the supplementation of dissociated Day 31+ hESC-derived cultures with Y-27632 leads to increases in human motor neuron numbers peaking at Day 31+9, through enhancement of proliferation of motor neuron progenitors and increased human motor neuron survival. Less is known about the effects of Y-27632 on motor neuron yields when added during critical periods of motor neuron differentiation from hESCs. In order to determine if Y-27632 supplementation during motor neuron differentiation and prior to dissociation is able to impact final motor neuron yields, we used the Hb9::GFP hESC reporter line and employed a standard optimized motor neuron differentiation protocol relying on retinoic acid (RA) and recombinant sonic hedgehog (SHH). Cells were cultivated under three differentiation conditions: CONTROL (standard protocol), Y-27632 treatment from day 11 until day 31 (Y-27632 D11-D31) and Y-27632 supplementation from day 22 until day 31 (Y-27632 D22-D31). Final motor neuron

abundance was determined through quantification of the GFP-positive fraction using optimized FACS sorting conditions. The addition of Y-27632 to the motor neuron-committed hESC cultures from day 11 onwards or Y-27632 supplementation during the last 10 days of motor neuron differentiation (day 22 until day 31) did not change significantly the motor neuron differentiation yield. Together, our data suggests that Y-27632 supplementation during critical periods of motor neuron differentiation is not a robust strategy to enhance final motor neuron yields.

We are grateful to NINDS, P^2ALS, Project A.L.S., NYSTEM, FCT and FLAD for supporting this work.

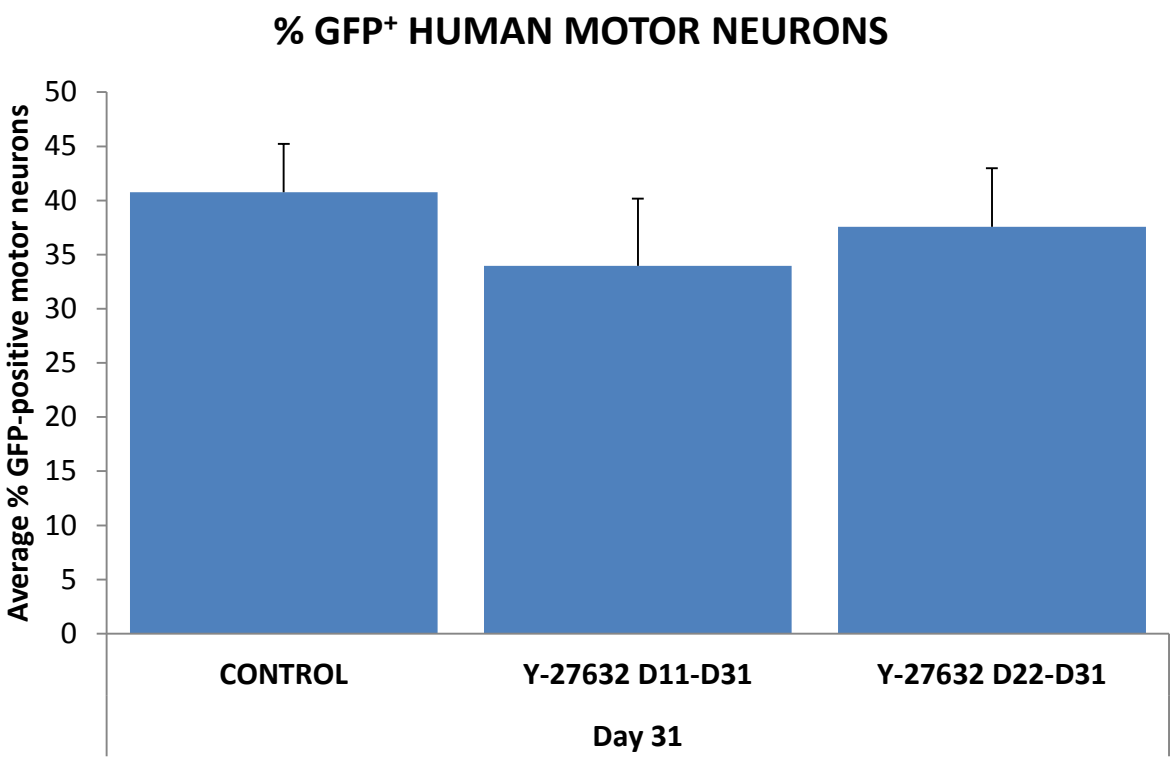

FIGURE 1. The presence of 10 µM Y-27632 during critical periods of motor neuron specification from the Hb9::GFP hESC line did not lead to enhanced motor neuron yields. Continuous 10 µM Y-27632 supplementation of motor neuron-committed hESC cultures during 10 (Y-27632 D22-D31) or 20 days (Y-27632 D11-D31) did not significantly impact on the overall motor neuron percentages at Day 31, as quantified using FACS sorting. The Hb9::GFP hESC reporter line was used to facilitate Hb9 motor neuron visualization in culture. A standard optimized differentiation protocol using retinoic acid (RA) and recombinant sonic hedgehog (SHH) was used to drive motor neuron differentiation from hESCs. Cells were cultivated under three differentiation conditions: CONTROL (standard protocol), 10 µM Y-27632 treatment from day 11 until day 31 (Y-27632 D11-D31) and 10 µM Y-27632 supplementation from day 22 until day 31 (Y-27632 D22-D31). Values are mean ± s.e.m., *n*=3 (t-test, $p>0.05$).

CHAPTER 3.3

Nuno Jorge Lamas, Hynek Wichterle and Christopher E. Henderson

The effects of Y-27632 on hESC-motor neuron generation and survival involve ROCK-independent mechanisms

(*Abstract*)

2014

The effects of Y-27632 on hESC-motor neuron generation and survival involve ROCK-independent mechanisms

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ABSTRACT

Human embryonic stem cells (hESCs) offer the possibility to create *in vitro* unlimited sources of cell types that are hard to obtain by biopsy, including the spinal cord motor neurons. Rho-ROCK is a conserved cellular pathway involved in crucial aspects of cell behaviour like motility, division, apoptosis and neurite outgrowth. ROCK inhibitor small molecules are promising compounds displaying interesting features in different neuronal cells types, including cell proliferation, survival and neurite outgrowth. We previously demonstrated that Y-27632, a widely-studied member of this family of compounds, was capable to enhance motor neuron generation and motor neuron survival in dissociated Day 31+ hESC-derived motor neuron cultures. Here, to better understand whether the inhibition of the Rho-ROCK pathway was responsible for the effects induced by Y-27632 on hESC-MN cultures we tested if other compounds belonging to the ROCK inhibitor family could induce similar effects in hESC-derived motor neuron cultures. Eight different ROCK inhibitor molecules (Y-27632, Fasudil, HA-1100, H-1152, Y-39983, GSK429286, SR3677 and Thiazovivin) were for the first time tested side-by-side using three newly developed assays involving hESC-derived motor neurons: mixed culture survival/proliferation assay, survival assay and neurite outgrowth assay using purified human motor neurons. Each compound was tested at serial dilutions in the 0.1 μM–100 μM concentration range. We observed that

Y-27632 was the only ROCK inhibitor molecule tested to display the capacity to significantly increase motor neuron numbers in mixed hESC-derived motor neuron cultures, in a dose-dependent manner. In addition, Y-27632 could also significantly increase the survival of purified human motor neurons deprived of neurotrophic factors for nearly 7 days. Y-39983, GSK429286 and Thiazovivin also showed a mild motor neuron survival-inducing effect, but the significant effects were only registered in three out of the seven concentrations tested. Interestingly, all eight molecules were capable of significantly boosting motor neuron axonal outgrowth, in line with previous studies showing that ROCK inhibition enhances neurite outgrowth. Taken together, our data suggest that Y-27632 is capable of promoting robust motor neuron generation and motor neuron survival in hESC-derived motor neurons likely by a ROCK-independent mechanism.

We are grateful to NINDS, P^2ALS, Project A.L.S., NYSTEM, FCT and FLAD for supporting this work.

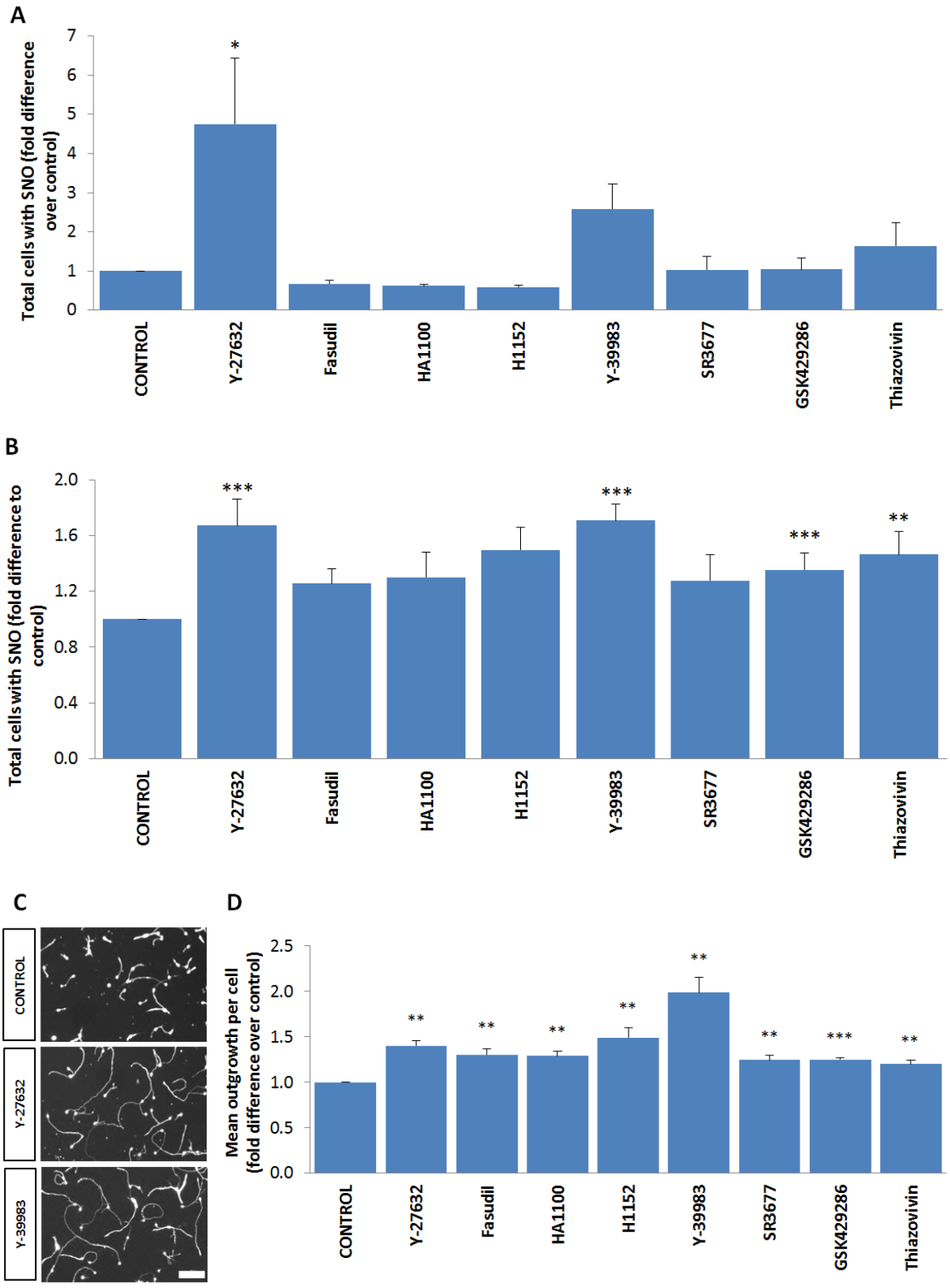

FIGURE 1. Y-27632 promotes motor neuron generation and motor neuron survival in hESC-derived motor neuron cultures likely by a ROCK-independent mechanism.

(A) Survival/proliferation assay using a mixed culture of progenitor cells and post-mitotic hESC-MNs dissociated at Day 31. Different ROCK inhibitors where tested for their capacity to

increase human MNs in culture. Readouts were performed at Day 31 + 9. Per compound, data is normalized to the average number of cells with significant outgrowth in control conditions (absence of drug). The best effect in human motor neuron number increase per compound is shown. Each compound was tested at different serial dilutions in the 0.1 µM–100 µM concentration range. Only Y-27632 showed the capacity to significantly increase human motor neuron numbers in culture. Values are Mean ± SEM, for $n \geq 3$ (t-test, *$p<0.05$).

(B) Survival assay using a FACS-purified population of human motor neurons. Different ROCK inhibitors where tested for their capacity to increase the survival of human motor neurons in culture. Readouts were performed seven days after cell seeding. Per compound, data is normalized to the average number of cells with significant outgrowth in control conditions (absence of drug). The best effect in motor neuron survival per compound is shown. Each compound was tested at different dilutions in the 0.1 µM–30 µM concentration range. Only Y-27632 showed the capacity to significantly increase the survival of human motor neurons submitted to neurotrophic deprivation for nearly 7 days (significant results in 5 out of the 7 concentrations tested). Three other small molecules (Y-39983, GSK429286 and Thiazovivin) also displayed a mild motor neuron survival-inducing effect (significant effects in 3/7 concentrations tested). Values are Mean ± SEM, for $n \geq 3$ (t-test, **$p<0.01$; ***$<p<0.001$).

(C) Effects of the indicated ROCK inhibitors on axonal outgrowth from purified human motor neurons at Day 31+2, stained using the vital dye calcein-AM that fills all processes. Scale bar = 50 µm.

(D) Neurite outgrowth using FACS-purified post-mitotic hESC-MNs. Neurite outgrowth is known to be enhanced by ROCK inhibition. Different ROCK inhibitors where tested for their capacity to increase neurite outgrowth as a proof of principle for ROCK inhibition. Readouts were performed two days after cell seeding. Per compound, data is normalized to the average mean outgrowth per cell in control conditions (absence of drug). The best effect in motor neuron neurite outgrowth increase is shown per compound. Each compound was tested at different dilutions in the 0.1 µM–30 µM concentration range. All the compounds to a lesser or greater extent induced a significant increase in the neurite outgrowth of human motor neurons. This suggests that all ROCK compounds tested are active at inhibiting the ROCK pathway, even though only Y-27632 robustly increased motor neuron numbers in culture. Values are Mean ± SEM, for $n \geq 4$ (t-test, **$p<0.01$; ***$<p<0.001$).

CHAPTER 3.4

Kevin C. Kanning, Hai Li, Elena Nikulina, Jianwi Hou, Wan S, Yang, Artem Kaplan, John R. Bermingham, Nuno Jorge Lamas, Mackenzie W. Amoroso, Hynek Wichterle, Marie T. Filbin, Brent Stockwell and Christopher E. Henderson

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Making motor axons grow

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ABSTRACT

The axons that connect motor neurons to their target muscles are among the longest single structures in the adult body. Even in the embryo, to innervate a muscle in the distal part of the limb a motor neuron needs to generate a process that is >200 cell diameters in length. The quantitative challenge is greater still for an injured adult axon that may need to regenerate over tens of centimeters. Therefore identifying mechanisms that make motor axons longer is critical both for understanding how the neuromuscular system is initially wired and, potentially, for enhancing its regeneration. We are studying intrinsic mechanisms that stimulate motor axon outgrowth.

Classical transplantation studies show that, even if the order of segments in the limb is surgically altered, axons from a given motor pool grow out the same distance as they would in control embryos. This, and other data for cranial sensory ganglia, suggests that there are intrinsic determinants of axon length but the underlying molecular code has not been elucidated. We found that in mouse embryos the transcription factor POU3F1/SCIP is selectively expressed in motor neurons innervating distant muscle targets (diaphragm, distal forelimb and distal hindlimb) but not in pools innervating more proximal muscles. In *pou3f1* null embryos, whereas all proximal muscles were normal, none of the targets of the POU3F1-expressing motor neurons ever became fully innervated, and motor neurons whose axons failed to reach their target subsequently died. POU3F1 therefore triggers a program for innervation of distant muscle targets that is common to motor pools at multiple rostrocaudal levels.

To define the signaling pathways underlying axonal growth, we screened a 50,000-compound library using an *in vitro* assay in which mouse or human ES cell-derived motor neurons are grown on inhibitory substrata. The most potent hits were the cholesterol-lowering drugs statins. When applied to human ES cell-derived motor neurons they induce a 5-fold increase in axon growth over the first 20 h. *In vivo*, they enhance optic nerve regeneration 5-fold over controls. Statins stimulate motor axon growth by inhibiting HMG-CoA reductase and thereby downstream protein prenylation. Since HMG-CoA reductase is expressed in motor neurons at high levels, it may potentially play a role in inhibiting axonal growth in multiple contexts.

Overall, these studies show that a combination of transcriptional and post-translational mechanisms govern motor axon length and suggest that they may be potential therapeutic targets in situations requiring stimulation of axonal growth.

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CHAPTER 3.5

Nuno Jorge Lamas, Sofia Serra, António J. Salgado, Nuno Sousa

Failure of Y-27632 to improve the culture of adult human adipose-derived stem cells

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ORIGINAL RESEARCH

Failure of Y-27632 to improve the culture of adult human adipose-derived stem cells

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Abstract: Y-27632 is a well-known inhibitor of the Rho-associated coiled kinase (ROCK) and has been shown to significantly improve the culture of a variety of multipotent stem cell types. However, the effects of Y-27632 on the expansion of adult human adipose-derived stem cell (hADSC) cultures remain to be established. Here, we aimed to characterize the effects of Y-27632 on the culture of hADSCs. Adult hADSCs were isolated from subjects submitted to elective plastic surgery procedures and cultivated in vitro under optimized conditions. Our results show that the continuous supplementation of hADSC cultures with Y-27632 led to decreased numbers of cells and decreased global metabolic viability of hADSC cultures when compared with control conditions. This effect appeared to be dependent on the continuous presence of the drug and was shown to be concentration-dependent and significant for 10 μM and 20 μM of Y-27632. Moreover, the Y-27632-induced decrease in hADSC numbers was not linked to a block in global cell proliferation, as cell numbers consistently increased from the moment of plating until passaging. In addition, Y-27632 was not able to increase the number of hADSCs present in culture 24 hours after passaging. Taken together, our results suggest that, in contrast to other stem cell types, Y-27632 supplementation is not a suitable strategy to enhance hADSC culture expansion.

Keywords: human mesenchymal stem cells, human multipotent stromal cells (hMSCs), ROCK inhibitor, MTS assay

Introduction

Stem cells hold the capacity to self-renew and to differentiate into tissue-specific cell types. Over the last decades, the identification of different populations of stem cells has attracted the interest of the scientific community due to their enormous potential, including applications in the field of regenerative medicine and drug discovery.[1–3] Strategies for efficient culture of these stem cells have long been pursued, rendering the procedures for stem cell culture less complex over time.[4,5] A recently identified small molecule that has been proven to be particularly advantageous in stem cell culture is Y-27632, a selective inhibitor of the Rho-associated coiled kinase (ROCK).[6,7] This compound has been demonstrated to display pleiotropic and wide-range positive effects across different cells types, namely human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).[6,7] Several reports have established that treating cultures of adult and human embryonic stem cells with Y-27632 considerably increases the post-thaw cell viability, cell attachment, and post-passaging viability, in addition to significant improvements in cellular proliferation.[6–10] However, the influence of Y-27632 in the culture of adult human adipose-derived stem cells (hADSCs)

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has not yet been thoroughly assessed. The hADSCs constitute a population of adult stem cells easily isolated from body tissues containing fat.[11,12] Similarly to other stem cell types, hADSCs retain the capacity to self-renew and to differentiate into diverse cell types, namely adipocytes, osteoblasts, chondrocytes, myocytes, cardiomyocytes, and neuron-like cells.[12–14] Due to their differentiation potential, simplicity to isolate, and ability to proliferate in vitro, the scientific community consider the hADSCs as an increasingly attractive source of patient-specific stromal/stem cells.[15–17]

Here, we set out to investigate if the supplementation of hADSC cultures with Y-27632 would significantly improve the culture of this population of adult multipotent stromal/stem cells. Our results demonstrate a Y-27632-induced decrease in hADSC number and global metabolic viability upon continuous Y-27632 supplementation, in comparison with control conditions. The effect was shown to be concentration-dependent (significant effect for 10 μM and 20 μM of Y-27632) and also dependent on the continuous presence of the drug. Interestingly, cell numbers increased from the moment of plating until passaging, so that the Y-27632-induced decrease in hADSC numbers was not linked to a block in global cell proliferation. In addition, Y-27632 could not increase the number of hADSCs present in culture 24 hours after passaging. Together, our results suggest that Y-27632 supplementation alone is not an appropriate strategy to significantly improve the culture conditions of adult hADSCs.

Materials and methods

Adult human adipose-derived stem cells isolation

Adult hADSCs were kindly provided by Jeffrey M Gimble (Pennington Biomedical Research Center, Baton Rouge, LA, USA). Cells were isolated according to a protocol previously described by Dubois et al.[18] Briefly, liposuction aspirates from subcutaneous adipose tissue were obtained from patients submitted to elective plastic surgery procedures. All donors gave their written informed consent. Tissues were then digested in a 0.1% collagenase type I solution (Worthington Biochemical Corporation, Lakewood, NJ, USA) pre-warmed to 37°C for 60 minutes. Afterward, the digested tissue was centrifuged for 5 minutes at 300–500 *g* at room temperature. The supernatant, containing mature adipocytes, was aspirated. The pellet was identified as the stromal vascular fraction (SVF). The SVF was resuspended and plated in T225 flasks in Stromal Medium (Dulbecco's Modified Eagle's Medium [DMEM]/Ham's F-12, 10% fetal bovine serum [FBS; Hyclone, Logan, UT, USA], 100 U penicillin/100 μg streptomycin/0.25 μg Fungizone®) at a density of 0.156 mL of tissue digest/cm^2 of surface area for expansion and culture. After reaching confluency, cells were passaged and kept in stromal medium.

Adult human adipose-derived stem cells culture and survival/proliferation studies

In this work, we used hADSCs previously cultured and cryopreserved between p5 and p10. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2. After quickly thawing the vials in a 37°C water bath, cells were resuspended in culture medium containing Minimum Essential Medium α (α-MEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 units/mL penicillin (Thermo Fisher Scientific), and 100 μg/mL streptomycin (Thermo Fisher Scientific). Cells were then spun at 1,200 *g* for 5 minutes. The supernatant was gently aspirated and cells resuspended in the same culture medium and plated in a T75 flask and allowed to expand. Media was changed every 3–4 days. Upon confluency, cells were trypsinized and passaged to new T75 flasks or plated in 6-, 12-, or 24-well plates (Nunc; Thermo Fisher Scientific) for the survival/proliferation studies. These survival/proliferation studies were carried out using cells plated at two initial seeding densities (1,000 and 5,000 cells/cm^2). Y-27632 was added to the wells within the first 60 minutes after cell plating. Throughout the study, medium was changed in all conditions at selected time points. Cells were fixed at the desired time points (24 hours and 5 days post-plating). In each study, 2–3 replicate wells were used per studied condition.

Y-27632

The putative ROCK inhibitor Y-7632 was purchased from Abcam (ab120129; Cambridge, UK) and dissolved in deionized filtered water to yield a 10 mM stock solution. Aliquots were prepared and stored at –20°C.

Cell viability assessment

Cell metabolic viability was assessed by performing MTS assays. The MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium test. The MTS test (Promega Corporation, Fitchburg, WI, USA) is a cell viability assay based on the bioreduction of the substrate (MTS) to a brown formazan product. After the desired cell culture period, the medium was aspirated and cells replenished with a new serum-free medium containing MTS in a 5:1 ratio and incubated at 37°C with 5% humidified

CO_2. Three hours post-incubation, 150 µL of each sample were transferred to 96-well plates (n=3 or 4) and optical density (OD) at 490 nm was determined using a Model 680 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Cell proliferation

The cellular proliferation of hADSCs was determined by a colorimetric assay based on 5-bromo-2′-deoxyuridine (BrdU) incorporation (Hoffman-La Roche Ltd, Basel, Switzerland). Cells were incubated with BrdU over 48 hours. After the BrdU incubation period, an enzyme-linked immunosorbent assay (ELISA) test was performed according to the manufacturers recommended protocol. In the end, the OD was determined at 370 nm with a reference filter at 492 nm using an Infinite 200 PRO NanoQuant Reader (Tecan Group Ltd, Männedorf, Switzerland).

Immunocytochemistry

Adult hADSCs cultures were pre-fixed by adding one volume of cold 4% paraformaldehyde diluted in phosphate-buffered saline 1× (PBS 1×/4% PFA) for 2 minutes at room temperature. Then, cells were fixed with PBS 1×/4% PFA for 30 minutes at 4°C. After fixation, cells were washed with PBS 1× three times and then incubated in 0.1 µg/mL of red phalloidin (Sigma-Aldrich Co, St Louis, MO, USA) diluted in PBS 1× for 1 hour at room temperature. In the end, cells were again washed with PBS 1× and finally incubated in a solution containing 1 µg/mL 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) for 20 minutes. Cells were washed again with PBS 1× and then imaged.

Image acquisition and quantitative image analysis

Image acquisition was performed using an IX-81 fluorescence microscope (Olympus Corporation, Tokyo, Japan). Seven representative fields of each well were selected in a blinded and systematic procedure using 10× magnification. Automated quantitative determination of DAPI-positive nuclei was performed using the Cell Scoring module of Metamorph NX software (Molecular Devices LLC, Sunnyvale, CA, USA). Positive cells were selectively identified when having clear signal intensity above local background. Intensity thresholds were set blinded to sample identity and in a given experiment, identical parameters were used in all images analyzed. The parameters were minimally adjusted across the different independent experiments.

Statistical analysis

All quantitative data were analyzed using GraphPad Prism for Windows (v5.00; GraphPad Software, San Diego, CA, USA). For each set of data, each condition was compared using one-way analysis of variance (ANOVA) statistical evaluation followed by Tukey's honestly significant difference (HSD) post hoc test. In cases involving only one time point and a two-group comparison, *P*-value was determined using Student's *t*-tests. Differences were considered to be significant when $P<0.05$.

Results

The expansion of hADSCs is not enhanced in the presence of Y-27632

Several reports in the last decade have demonstrated that Y-27632 efficiently improves the culture conditions of a myriad of human stem cell types, including hESCs, hiPSCs, human mesenchymal stem cells (hMSCs) and human neuronal progenitor cells (hNPCs).[6,7,9,10,19–23] However, the influence of Y-27632 in the culture of adult hADSCs remains to be firmly established. Here, we first aimed to assess if the presence of Y-27632 in the culture medium could improve the overall expansion of hADSCs. Human ADSCs p5 and p10 were grown in T75 flasks until confluency (Figure 1A). Afterward, cells were trypsinized and the resultant cell suspension recovered to be replated in 12-well plates. The pilot experiments performed showed that hADSC cultures seeded initially at the standard 5,000 cells/cm^2 seeding density would reach confluency after 5 days of continuous culture (control hADSC growth condition; Figure 1B). Thus, all subsequent expansion experiments were conducted until day 5 after hADSC plating. To evaluate the effect of Y-27632 in hADSCs, these adult stem cells were grown under continuous standard 10 µM Y-27632 supplementation for 5 days (Y-27632 D0–D5); Y-27632 treatment for the first 24 hours followed by culture in regular growth conditions for the next 4 days (Y-27632 D0–D1); or growth in control conditions (control; Figure 1A). Two initial cell-seeding densities (1,000 and 5,000 cells/cm^2) were used (Figure 1A and B). During the 5-day period, a continuous increase in the number of hADSCs was registered across the three different medium supplementation conditions. At day 5, the cell cultures were fixed with PFA and stained for quantification of the number of hADSCs present in the culture. Using 10× magnification, seven representative fields of each well were then imaged in a blinded and systematic manner (Figure 2A). Automated quantitative image analysis of hADSCs present in the wells

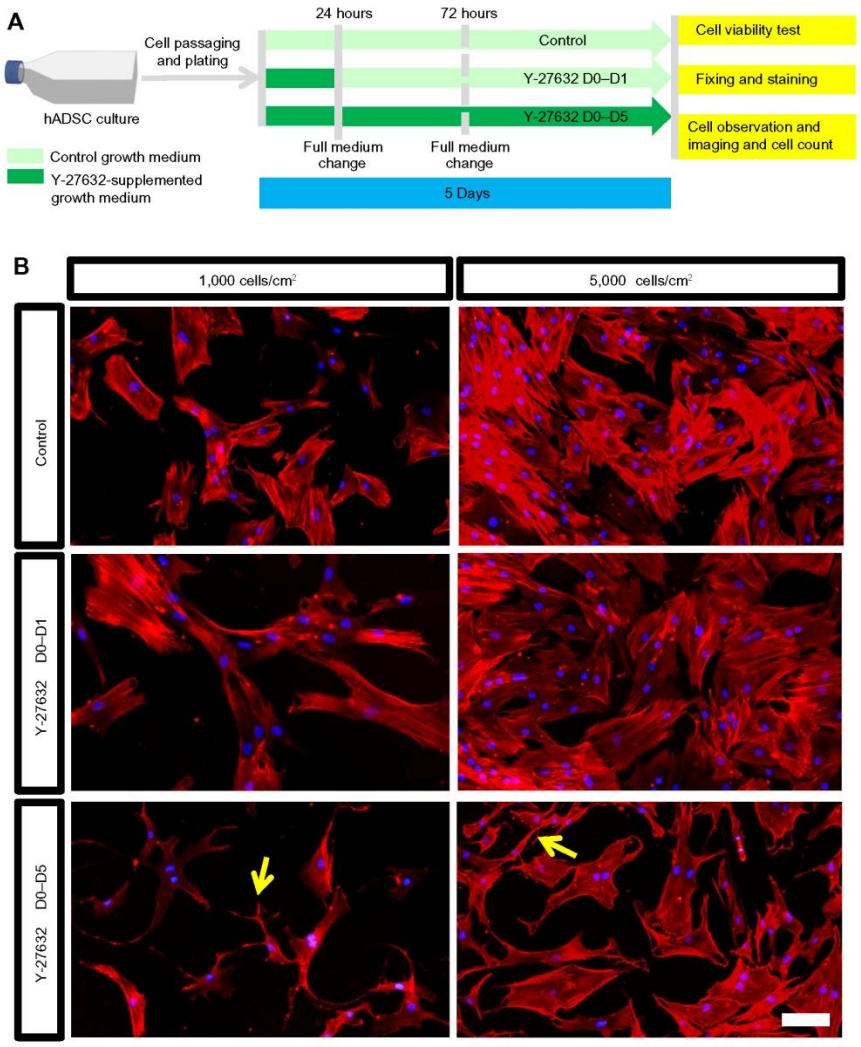

Figure 1 The continuous presence of 10 μM Y-27632 did not lead to enhanced hADSC expansion.
Notes: (**A**) Diagram of the protocol used to study the effect of Y-27632 in hADSCs cultured over 5 days. Two initial seeding densities (1×10^3 and 5×10^3 cells/cm^2) were used for these studies. Cells were grown in control growth medium for 5 days (Control); Y-27632 treatment for the first 24 hours and then growth in control medium for the next 4 days (Y-27632 D0–D1); or continuously cultivated in the presence of 10 μM Y-27632 for 5 days (Y-27632 D0–D5). (**B**) Representative images of hADSC cultures after 5 days of continuous culture. None of the treatments could halt cellular proliferation, and therefore, cell numbers increased significantly from day 1. Uninterrupted supplementation of hADSCs with 10 μM Y-27632 led to a lower cell number at day 5 comparative to control conditions. A few cells grown under the continuous influence of Y-27632 evidenced a neuronal-like morphology after 5 days (yellow arrows), but failed to demonstrate robust expression of putative neuronal markers. Scale bar = 80 μm.
Abbreviation: hADSCs, human adipose-derived stem cells.

was then performed using the Cell Scoring module of the Metamorph NX software (Figure 2B). After 5 days of cell culture, we registered similar numbers of hADSCs in the control and Y-27632 D0–D1 wells (Figures 1B, 2C, and 2D). On the other hand, Y-27632 D0–D5 wells had significantly decreased numbers of hADSCs when compared to the control and Y-27632 D0–D1 wells (Figures 1B, 2C, and 2D). The analysis of the global cell metabolic viability using the MTS test revealed significantly decreased OD in hADSCs grown continuously in the presence of Y-27632 (Y-27632 D0–D5; Figure 2E and F). These results were observed for both seeding densities tested (Figure 2C–F). Remarkably, the number of hADSCs present in culture was also diminished even if the Y-27632 treatment was continuously applied over 4 days from day 1 to day 5 (data not shown). Interestingly, at day 5, we observed that a few cells treated with Y-27632 over 5 days exhibited neuron-like morphology (Figure 1B; Y-27632 D0–D5). However, those cells failed to express

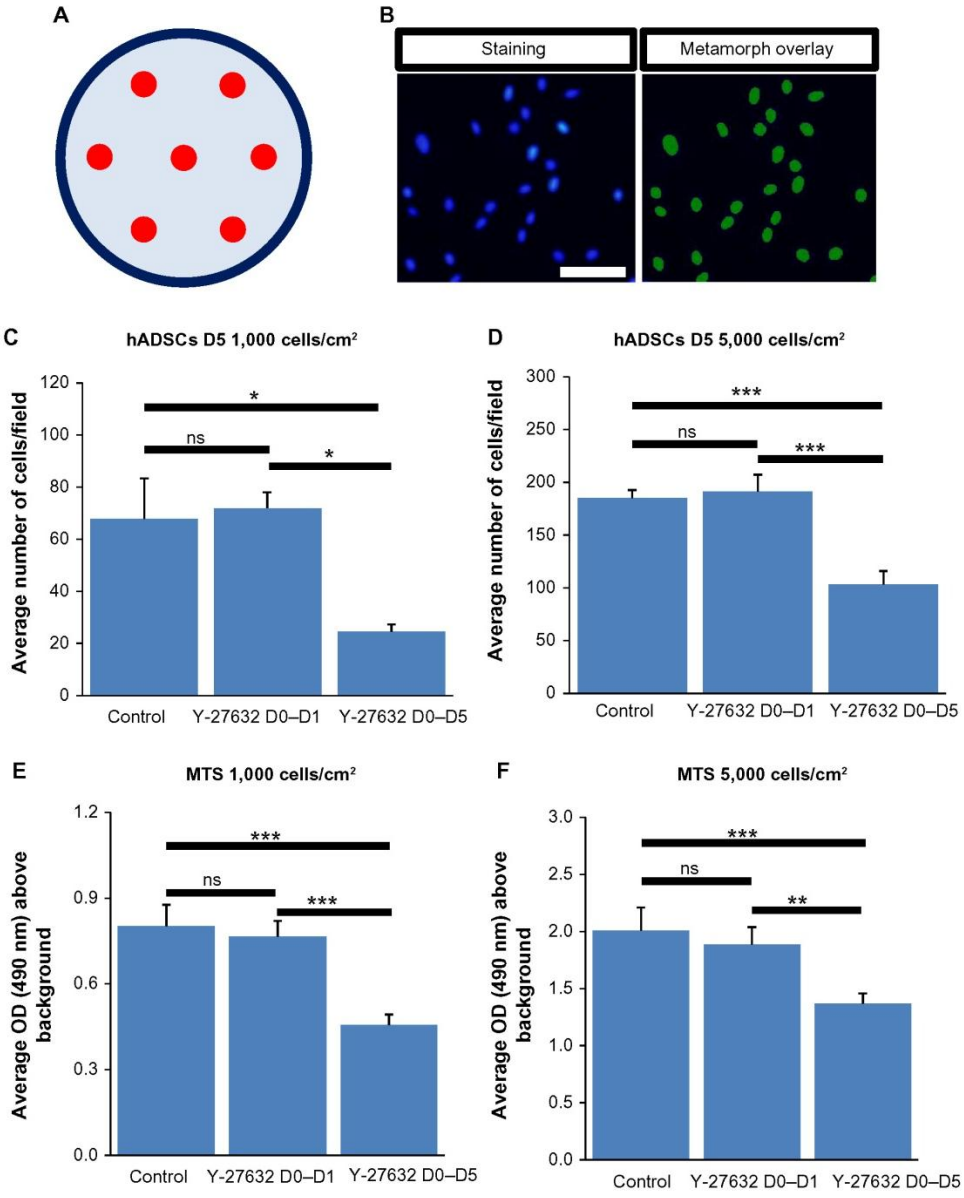

Figure 2 Cell number and cellular viability of hADSC cultures at day 5.

Notes: (**A**) Image acquisition for cell number quantification was performed in a blinded procedure through acquisition of images at 10× magnification in seven systematic regions (red dots) in each well studied. (**B**) Sample image of DAPI-stained hADSCs and corresponding overlay for automated quantitative determination of DAPI-positive nuclei using the Cell Scoring module of Metamorph NX software. Scale bar =110 µm. (**C**) Continuous Y-27632 supplementation attenuated the expansion of hADSCs seeded initially at 1×10^3 cells/cm² and (**D**) 5×10^3 cells/cm². Values shown as mean ± SEM, n=5 (***$P<0.001$, *$P<0.05$, ns = not significant). (**E**) The sustained treatment with 10 µM Y-27632 led to decreased cellular viability in hADSCs seeded initially at 1×10^3 cells/cm², and (**F**) 5×10^3 cells/cm² when compared to control conditions. Values shown as mean ± SEM, n=5 (***$P<0.001$, **$P<0.01$, ns = not significant).

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; hADSCs, human adipose-derived stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium; OD, optical density; SEM, standard error of the mean.

putative neuronal markers, namely the microtubule-associated protein 2 (MAP-2) as assessed by immunocytochemistry performed under optimized conditions in three independent experiments. Taken together, these results highlight that the continuous presence of Y-27632 in hADSC cultures could lead to decreased numbers of cells in culture when compared to control conditions, in contrast to what has been widely demonstrated in other stem cell types.

The effect of Y-27632 in hADSC cultures is concentration-dependent

To further confirm that the continuous presence of Y-27632 in culture does not enhance the expansion of hADSCs, we aimed to determine whether the effect of Y-27632 in hADSCs was concentration-dependent. Thus, we next performed studies involving serial concentrations of Y-27632 starting at 20 µM. For this set of experiments, hADSCs were seeded initially at 5,000 cells/cm^2 in 24-well plates. Three replicate wells were used for each concentration tested. At the end of the optimized 5-day culture period, we observed a robust concentration-dependent decrease in hADSC numbers in cultures treated continuously with Y-27632 (Figure 3A). The calculated half maximal effective concentration (EC_{50}) for the continuous Y-27632 treatment was determined to be ≈7.97 µM. The decrease in cell numbers was statistically significant for the concentrations 10 µM and 20 µM (Figure 3A). These concentrations of Y-27632 were associated with significant increases in the average nuclear area of hADSCs (Figure 3B) and a significant decrease in the global cellular metabolic viability (Figure 3C). Together, our results not only demonstrate a robust Y-27632

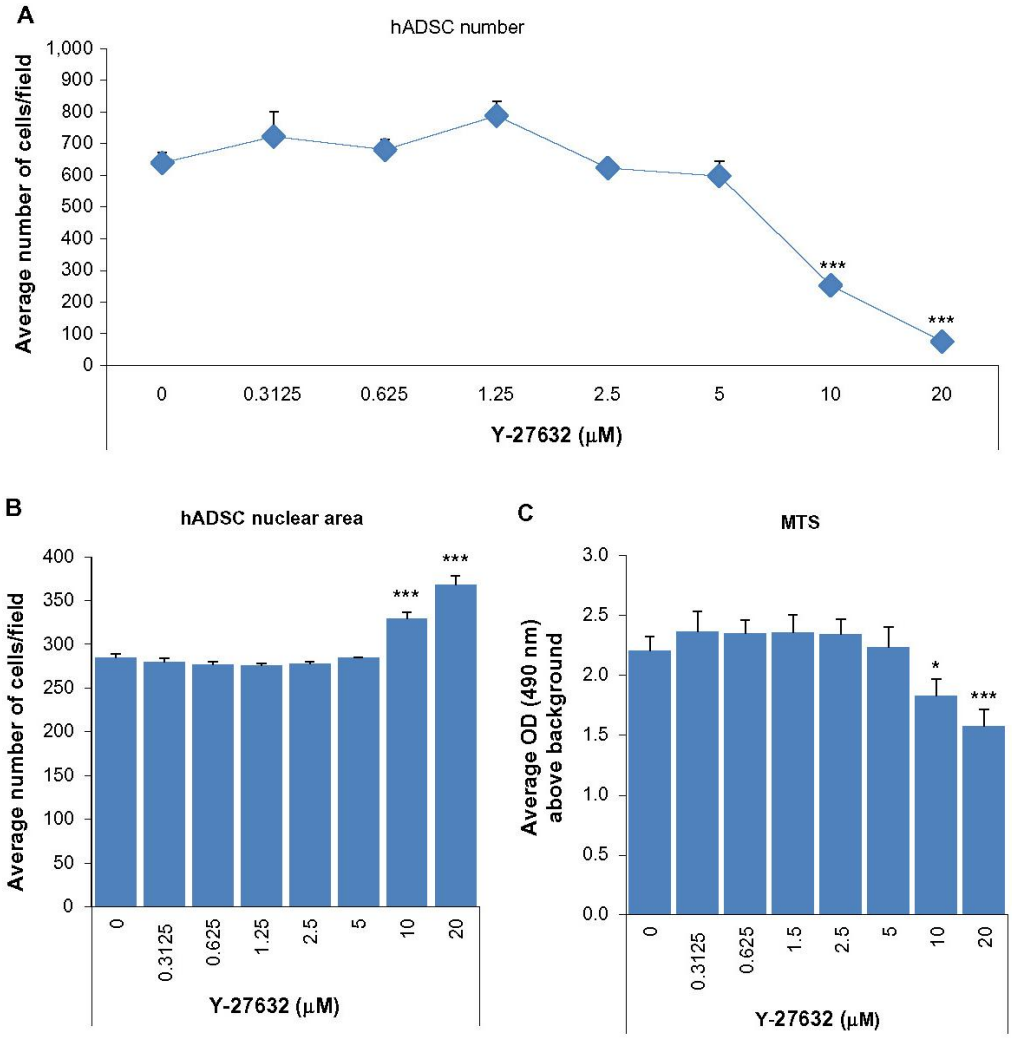

Figure 3 The effect of Y-27632 on the culture of hADSCs is concentration-dependent.

Notes: (**A**) Over 5 days, hADSCs were cultured under optimized conditions (without Y-27632) and in the presence of increasing concentrations of Y-27632. Cell numbers were significantly decreased when hADSCs were cultured with continuous 10 µM or 20 µM Y-27632 supplementation. Values shown as mean ± SEM, n=5 (***$P<0.001$). (**B**) hADSCs had significantly increased average nuclear area and (**C**) decreased global cell metabolic viability when cultured in the presence of 10 µM or 20 µM Y-27632. Values shown as mean ± SEM, n=5 (***$P<0.001$, *$P<0.05$).

Abbreviations: hADSCs, human adipose-derived stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium; OD, optical density; SEM, standard error of the mean.

concentration-dependent decrease in hADSC numbers, but also show for the first time that the standard 10 μM concentration of Y-27632, commonly associated with pro-expansion effects in most stem cell types, does not enhance the culture of hADSCs.

Effect of Y-27632 in the viability and continued culture of hADSCs

The initial experiments which demonstrated that continuous Y-27632 supplementation is not beneficial for hADSC culture were performed using cells in passage >6. It is possible that at this later passage, cells have lost their multipotency and have become nonresponsive to the standard 10 μM concentration of Y-27632. To rule out this possibility and to elucidate further the effect of Y-27632 in the hADSC cultures, we conducted a new set of experiments using hADSCs in earlier passages (<p4). hADSCs were again grown in T75 flasks until confluency (Figure 4A). Afterwards, cells were trypsinized and the resultant cell suspension recovered to be replated at 5,000 cells/cm^2 in 6-well plates. Cells were then allowed to expand over 5 days in the continuous presence or absence of the standard 10 μM Y-27632. After 5 days, cells were trypsinized and counted with the help of a hematocytometer. Our data showed decreased numbers of cells in the wells treated with Y-27632 for 5 days (Figure 4B), which shows that the decrease in hADSC number upon Y-27632 treatment is also observed in earlier passages of hADSCs.

Numerous reports demonstrate that Y-27632 treatment is associated with enhanced survival of recently passaged cells.[7,8,24,25] To determine if Y-27632 is also able to enhance the survival of hADSCs after cell passaging, cells grown for 5 days in the presence or absence of Y-27632 were replated and cultured for the next 24 hours with or without Y-27632 (Figure 4A and C). After 24 hours, we registered similar numbers of hADSCs in the untreated and Y-27632-treated wells (Figure 4C; ns = not significant). This observation was present in cells either grown with or without Y-27632 in the previous 5 days. Together, these results show that Y-27632 is not able to significantly increase the survival of recently passaged hADSCs.

We next aimed to assess the effect of prolonged Y-27632 supplementation on the continuous proliferation and viability of hADSCs. Those hADSCs grown initially over 5 days in the presence or absence of Y-27632 were passaged and allowed to re-expand for an additional 5 days (Figure 4A and D). After passaging, each initial group of cells was subsequently submitted to treatment with or without Y-27632 (Figure 4A and D). Therefore, hADSCs were grown under four different medium conditions (Figure 4A): 10 days of growth in control conditions (C/C); 5 days of growth in control conditions plus 5 days of continuous Y-27632 supplementation (C/Y); 5 days of growth with Y-27632 followed by growth in control conditions (Y/C); and 10 days of growth with Y-27632 treatment (Y/Y). We observed a continuous increase in the number of hADSCs in culture in both the first and the second period of 5 days of cell culture across the different growth conditions, which shows that Y-27632 treatment does not block the proliferation of hADSCs (Figure 4C vs 4D). After 10 days of culture, the wells submitted to a continuous treatment with Y-27632 during the second growth period had the lowest number of hADSCs (C/Y and Y/Y; Figure 4D). Contrarily, cells grown under control conditions in both periods had the greatest number of hADSCs in culture (C/C; Figure 4D). Interestingly, in the wells submitted to an initial 5-day treatment with Y-27632 and then a second period of growth in control conditions (Y/C), the number of hADSCs present in culture was similar to growth in control conditions for the 10 days (C/C; Figure 4D). In line with this, the global metabolic viability of hADSCs was decreased in the wells treated with Y-27632 for the last 5 days of cell growth (C/Y and Y/Y; Figure 4E). Yet, we did not register significant differences in the global levels of cell proliferation among the four growth conditions studied (Figure 4F). Together, this data reveals a long-term decrease in the number of hADSCs in the presence of Y-27632 when compared to control conditions. Our results also suggest that the removal of the drug from the growth medium allows cultures to re-expand up to the levels similar to that of the control growth condition, implying that the effects of Y-27632 on hADSC cultures are not irreversible, but seem to depend on the continuous presence of the drug.

The previous experiments were performed under a paradigm of continuous Y-27632 supplementation. To further confirm that the decrease in the number of hADSCs pertained by Y-27632 depends on continuous drug supplementation, we performed the same experiments under a paradigm of transient Y-27632 treatment during the 24 hours prior to cell passaging (Figure 5A). We observed that the 24 hours of treatment did not have a significant impact on the total number of cells retrieved from the wells after 5 days of hADSC expansion, resulting in similar total cell yields when compared to the control conditions (Figure 5A and 5B). In line with previous data, 24 hours after hADSC passaging, we registered similar numbers of hADSCs in the untreated and Y-27632-treated wells (Figure 5C), further confirming that Y-27632 does not seem to significantly enhance the survival of hADSCs. The hADSCs

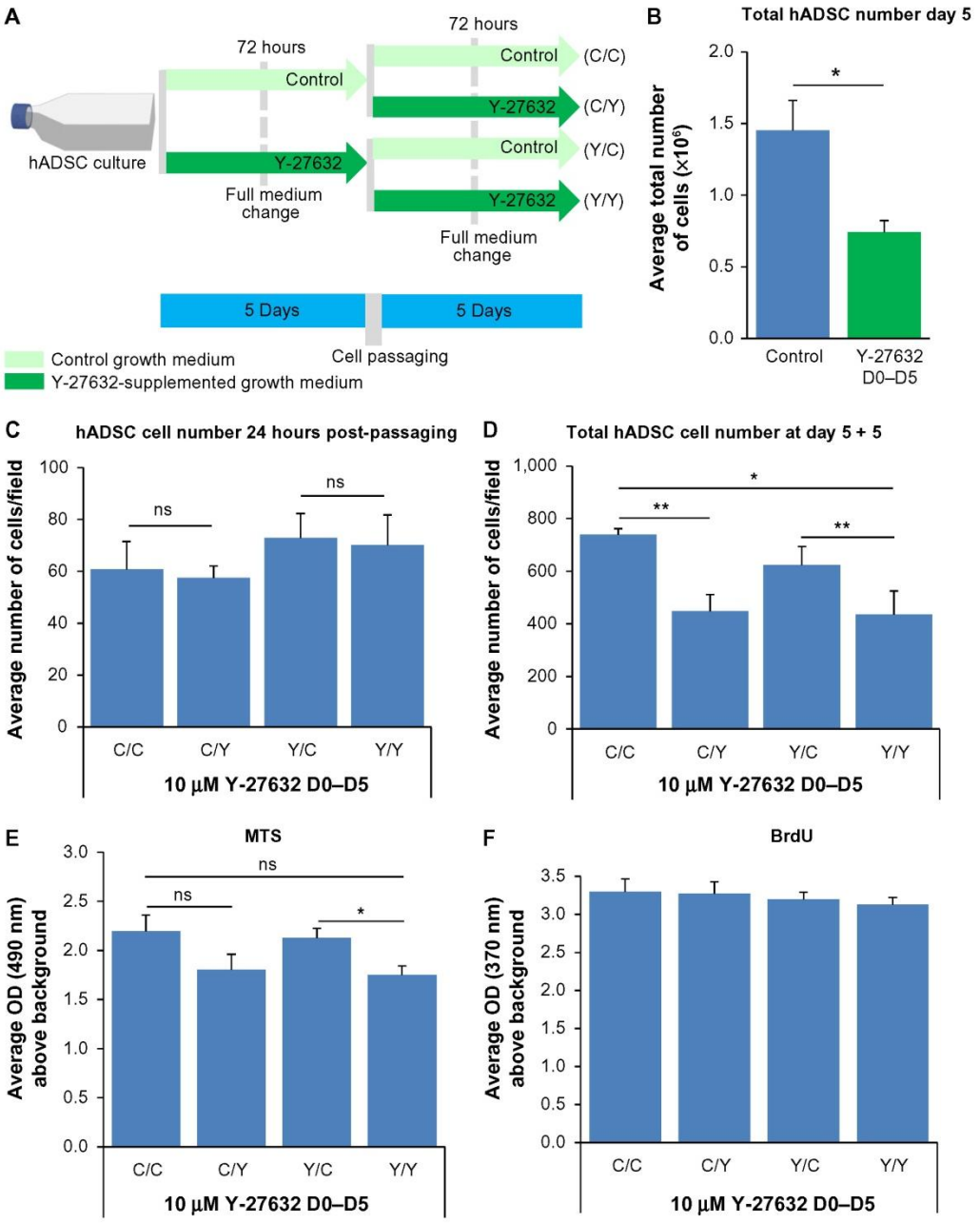

Figure 4 Prolonged Y-27632 supplementation is not associated with improvements in the expansion and survival of hADSCs.

Notes: (**A**) Diagram of the protocol used to study the effect of prolonged Y-27632 supplementation in early passage hADSC cultures. hADSCs were grown initially over 5 days in the presence or absence of Y-27632. Afterwards, cells were passaged and allowed to grow for an additional 5 days. The hADSCs from the two initial groups were each grown subsequently with or without Y-27632. (**B**) At the end of the initial 5 days of culture, continuous Y-27632 treatment led to systematically decreased numbers of hADSCs, even at early passages of hADSCs. Values shown as mean ± SEM, n=5 (*$P<0.05$). (**C**) Twenty-four hours after passaging, Y-27632 supplementation did not promote enhanced survival of hADSCs. This result was observed whether previous 5-day treatment with Y-27632 was present or absent. Values shown as mean ± SEM, n=4 (ns = not significant). (**D**) After 10 days of cell culture, hADSC numbers were significantly decreased in wells grown continuously with Y-27632 for the previous 5 days. An initial exposure to Y-27632 for 5 days followed by growth in control conditions did not preclude hADSCs to re-expand up to levels similar to the control conditions. Values shown as mean ± SEM, n=5 (**$P<0.01$, *$P<0.05$). (**E**) The continuous presence of Y-27632 was associated with decreased global cellular metabolic viability of hADSCs after 10 days of culture. Values shown as mean ± SEM, n=5 (*$P<0.05$, ns = not significant). (**F**) Global hADSC proliferation remained apparently unaffected by the presence of Y-27632, which is in line with the observed increase in cell numbers in both 5-day periods of cell culture studied. Values shown as mean ± SEM, n=3.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; hADSCs, human adipose-derived stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium; OD, optical density; SEM, standard error of the mean; C, control; Y, Y-27632.

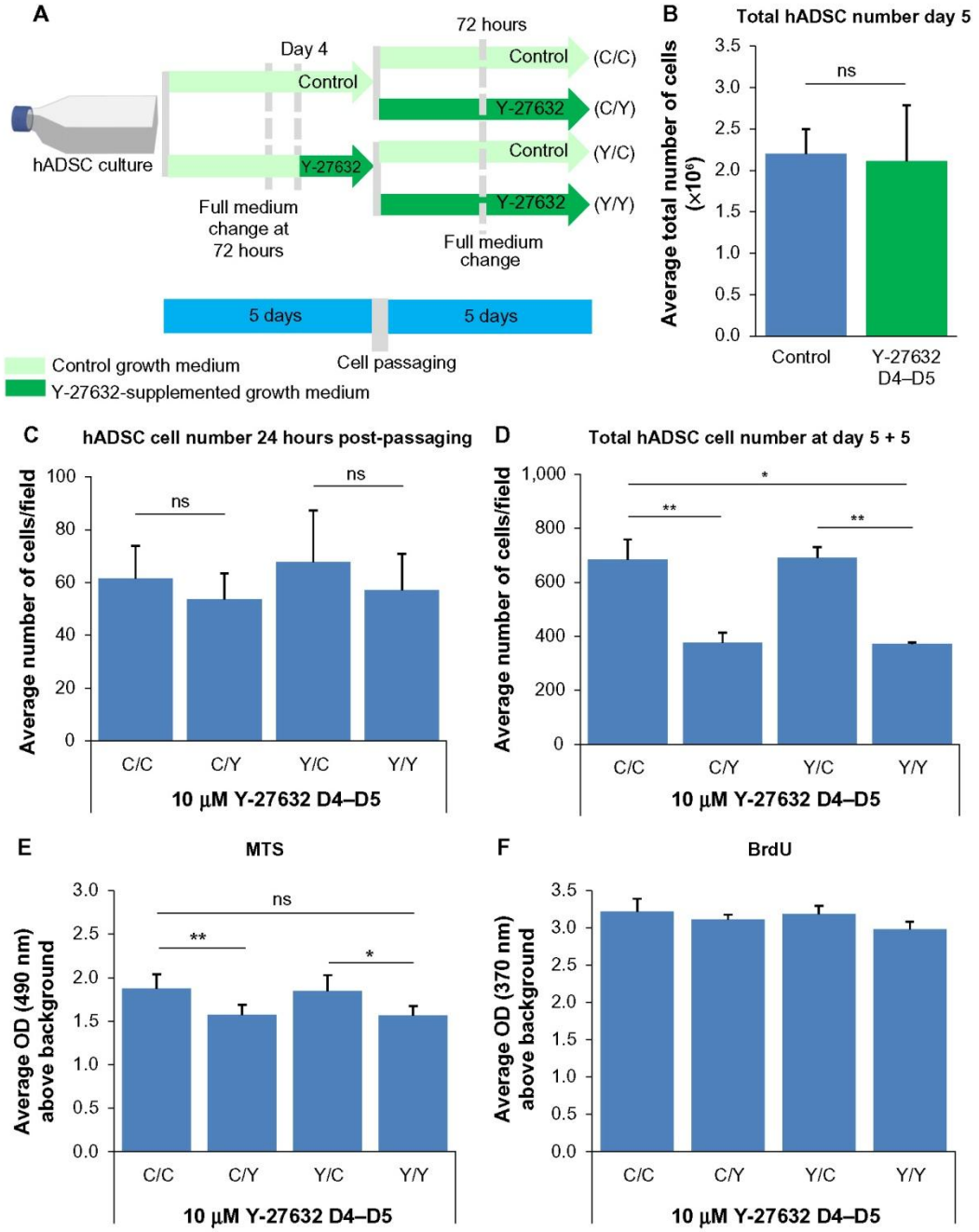

Figure 5 Y-27632 exposure over 24 hours prior to cell passaging was not associated with improvements in the expansion and survival of hADSCs.

Notes: (**A**) Diagram of the protocol used to study the effect of Y-27632 supplementation 24 hours before passaging in hADSC cultures. (**B**) At the end of the initial 5 days of culture, transient 24-hour exposure of hADSCs to Y-27632 treatment did not alter the number of cells retrieved upon passaging at day 5. Values shown as mean ± SEM, n=5 (ns = not significant). (**C**) Twenty-four hours after passaging, Y-27632 supplementation was not linked to enhancements in the survival of hADSCs. This result was observed whether treatment with Y-27632 in the preceding 24 hours was present or absent. Values shown as mean ± SEM, n=4 (ns = not significant). (**D**) After 10 days of cell culture, hADSC numbers were significantly decreased in wells grown continuously with Y-27632 for the previous 5 days. An initial exposure to Y-27632 for 24 hours followed by growth in control conditions did not preclude hADSCs to expand up to levels similar to the control conditions. Values shown as mean ± SEM, n=5 (**$P<0.01$, *$P<0.05$). (**E**) Continuous Y-27632 supplementation was associated with decreased global cellular metabolic viability of hADSCs after 10 days of culture. Values shown as mean ± SEM, n=5 (**$P<0.01$, * $P<0.05$, ns = not significant). (**F**) Global hADSC proliferation levels in the presence of Y-27632 were similar to control conditions, which is in line with the observed increase in cell numbers in both 5-day periods of cell cultures studied for control and Y-27632-treated wells. Values shown as mean ± SEM, n=3.

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; hADSCs, human adipose-derived stem cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium; OD, optical density; SEM, standard error of the mean; C, control; Y, Y-27632.

exposed to Y-27632 over the 24 hours before passaging were also replated and allowed to cultivate for an additional 5 days with and without Y-27632 (Figure 5A and 5D). As a result, the cells were again studied in four different medium conditions (Figure 5A): 10 days of culture in control conditions (C/C); 5 days of growth in control conditions plus 5 days of continuous Y-27632 supplementation (C/Y); 4 days of growth in control conditions followed by a 24-hour exposure to Y-27632; and then either growth in control conditions (Y/C) or 5-day growth submitted to Y-27632 (Y/Y). Here, we also observed a continuous increase in the number of hADSCs in culture in both the first and the second period of 5 days of cell culture across the different growth conditions (Figure 5C vs 5D). After 10 days of culture, the wells submitted to Y-27632 treatment during the second growth period had the lowest number of hADSCs (Figure 5D) and cells grown under control conditions for the entire 10-day period had the greatest number of hADSCs in culture (Figure 5D). Interestingly, in the wells where hADSCs were submitted to an initial 24 hours of Y-27632 treatment and then a second period of growth in control conditions (Y/C), the number of hADSCs present in culture was similar to growth in control conditions for the 10 days (C/C; Figure 5D). The global metabolic viability of hADSCs was decreased in the wells continuously treated with Y-27632 for the last 5 days of cell growth (Figure 5E). Interestingly, we did not register significant differences in the levels of cell proliferation among the four medium combinations tested (Figure 5F). This data further confirms that the continued decrease in the number of hADSCs in the presence of Y-27632 depends on the constant presence of the drug.

Taken together, the data reported in the present study demonstrate for the first time that continuous supplementation of hADSC cultures with Y-27632, even at the standard 10 μM concentration, does not constitute a beneficial strategy to improve the culture of hADSCs.

Discussion

The continuous addition of Y-27632 to stem cell cultures has been linked to considerable improvements in the expansion of some stem cell populations, specifically hPSCs and hNPCs.[6,23,26–28] We showed here that growing hADSC cultures in the presence of Y-27632 did not add benefits in terms of cell numbers and cell viability; moreover, the continuous presence of Y-27632 was shown to be detrimental for the expansion of this population of stem cells given that cell numbers and cell metabolic viability were significantly decreased in the different paradigms studied when compared to control conditions. However, the decrease in the number of hADSCs upon continuous treatment with Y-27632 was not linked to a block in cell proliferation, as cell numbers increased during 5 days and global BrdU levels were similar in wells treated with or without Y-27632. In addition, the removal of the Y-27632 compound from the medium allowed cells previously exposed to Y-27632 treatment (either 24 hours or 5 days continuously) to expand up to the levels of the control condition, which implies that the effects of Y-27632 on hADSC cultures reported here are not irreversible, but seem to depend on the continuous presence of the drug.[29] Therefore, it is plausible that Y-27632 is lengthening the progression of the dividing hADSCs through the cell cycle, which could explain overall lower hADSC numbers in comparison to control conditions, despite similar global levels of BrdU in the 48-hour pulse performed. Our results are in line with previous studies showing that the expansion of $CD34^+$ hematopoietic progenitor cells is impaired in the presence of Y-27632.[30,31] Both hADSCs and $CD34^+$ hematopoietic progenitor cells are adult stem cells of mesodermal origin and Y-27632 could be decreasing their expansion rates by using a common mechanism. The present study also showed that the application of Y-27632 to recently passaged hADSC cultures did not significantly increase the number of hADSCs 24 hours after passaging when compared to control conditions. This result is in contrast with studies involving other stem cells types, namely hPSCs, in which this compound is known to significantly increase cell survival of those stem cells upon dissociation due to its well-known antiapoptotic effects.[27,28] It is possible that the proapoptotic mechanisms activated in hPSCs upon dissociation differ from the possible proapoptotic mechanisms present in hADSCs in the first 24 hours after cell passaging. On the other hand, the molecular inhibition pertained by this compound in hADSCs (mesenchymal lineage) might lead to a diverse cellular phenotype from what has been widely described for hPSCs. Future studies are needed to thoroughly address the cellular mechanisms responsible for the diverse responses of different stem cell types to Y-27632.

The great majority of studies in the literature that demonstrate the beneficial effects of Y-27632 for cell proliferation and survival have been obtained using a standard 10 μM concentration of Y-27632. Here, we studied different concentrations of Y-27632 and showed that in wells grown continuously under 10 μM or 20 μM of Y-27632, there are significantly decreased numbers of hADSCs. Previous in vitro studies involving Y-27632 have established that this compound at 10 μM concentration, besides ROCK, can in addition strongly inhibit other kinases, namely PRK2, mitogen- and stress-activated protein kinase-1 (MSK1), leucine-rich repeat

protein kinase-2 (LRRK2), and MNK1.[32,33] This might explain the observed decrease in the number of viable cells at day 5 upon continuous Y-27632 supplementation, since the sustained inhibition of several protein kinases might be detrimental to cellular homeostasis in the long run.

A recent report in the literature has proposed that hADSCs treated with Y-27632 develop morphological changes resembling neuron-like cells.[34] Here, the continuous presence of Y-27632 led to the appearance of hADSCs with similar neuron-like shapes. We could demonstrate that these cells fail to express putative neuronal markers, namely MAP-2. Yet, it remains to be confirmed that Y-27632 alters the lineage commitment potential of hADSCs. Eventually, these morphological changes add another layer of evidence to highlight the negative value of adding Y-27632 to hADSC cultures, if the purpose is to culture this type of adult stem cells more efficiently.

Conclusion

The present work shows that Y-27632 supplementation not only does not increase the plating efficiency of hADSCs, but also fails to enhance the expansion of this type of human adult stem cells. In contrast to other stem cell types, Y-27632 supplementation does not seem to constitute a successful strategy to improve hADSC cultures.

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Author contributions

NJL: collection of data, conception and design, data analysis and interpretation, manuscript writing; SCS: collection of data, data analysis and interpretation, manuscript writing; AJS: conception and design, data analysis and interpretation, manuscript writing; NS: conception and design, data analysis and interpretation, manuscript writing. All authors read and approved the final manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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CHAPTER 4

DISCUSSION

4. DISCUSSION

The successful specification of many different cell types from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) represents an exciting new approach to unravelling the mechanisms of human embryonic development, and for drug discovery and modelling of diseases *in vitro*. Importantly, the ability to generate a specific cell type from human pluripotent stem cells (hPSCs) opens the possibility of creating inexhaustible sources of key cells that are otherwise inaccessible to study in the human body (Han et al., 2011; Nizzardo et al., 2010). This is the case of cells from the central nervous system, which have been classically hard to obtain due to ethical and technical constraints and hard to culture even when isolated (Faravelli et al., 2014; Han et al., 2011; Nizzardo et al., 2010; Palmer et al., 2001; Silani et al., 1998). Therefore, the differentiation of hPSCs into relevant human motor neuron populations has allowed mechanistic analysis of neuronal cell death and survival, as well as drug testing in human cellular models of motor neuron disease (Dimos et al., 2008; Ebert and Svendsen, 2010; Egawa et al., 2012; Faravelli et al., 2014; Han et al., 2011; Sareen et al., 2012; Wichterle and Przedborski, 2010). However, significant obstacles need to be overcome before the full potential of hPSCs to establish biochemical and screening approaches can be realized. On one hand, current protocols for motor neuron specification are lengthy and rely on costly recombinant growth factors, while motor neuron yields are suboptimal (Faravelli et al., 2014). On the other hand, our knowledge of the survival requirements of human motor neurons remains in its infancy. One appealing alternative source of stem cells capturing the genetic background of the donor is the human adipose tissue, from which human adipose-derived stem cells (hADSCs) can be easily isolated (Gimble and Guilak, 2003). However, current knowledge of the survival and expansion requirements of hADSCs is scarce and the ability to drive these cells efficiently towards a neuronal lineage remains to be developed.

In this work some of these problems were revisited and original strategies that allow us to significantly increase motor neuron yields from hPSC-derived cultures and to create a robust survival assay using human motor neurons specified from hPSCs were developed. Our initial studies involving hPSC-derived spinal motor neuron cultures revealed high levels of ongoing birth of new motor neurons. This finding was unexpected, and we decided to take the ongoing neurogenesis into account in two different ways. First, we

addressed the problem of insufficient motor neuron yields and screened a small collection of 160 bioactive molecules to find small molecules capable of increasing motor neuron numbers in culture. We identified the Rho-kinase (ROCK) inhibitor Y-27632 as an agent which could reliably increase motor neuron numbers up to four-fold after 9 days in culture. Second, to overcome the potential confound effect of ongoing neurogenesis on motor neuron survival studies we purified human motor neurons by FACS sorting and used them to create an assay for agents with direct effects on motor neuron survival. In line with previous studies in purified populations of chick and rodent embryonic motor neurons (Gould and Enomoto, 2009; Gould and Oppenheim, 2011; Henderson et al., 1998; Kanning et al., 2010; Thoenen and Sendtner, 2002), the human motor neurons were responsive to the survival-promoting actions of specific neurotrophic factors (GDNF, BDNF and CNTF), as well as the hit compound Y-27362 itself.

4.1. Human motor neurons are continuously generated in cultures derived from differentiated human induced pluripotent stem cells

During embryonic spinal cord development, motor neurons are generated from a limited pool of committed ventral spinal neuronal progenitors which express the transcription factor Olig2 (Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001). In rodents, these cells seem to be rapidly exhausted or converted to oligodendroglial progenitors (Marquardt and Pfaff, 2001; Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). In fact, Olig2-positive cells isolated from late embryos and transplanted to early chick neural tube were demonstrated to produce only oligodendrocytes (Mukouyama et al., 2006). Therefore, these motor neuron progenitors lose their potential to generate motor neurons over time and seem to be intrinsically programmed to generate only limited numbers of motor neurons (Mukouyama et al., 2006). Here, we discovered nearly incessant motor neuron production in cultures specified from hPSCs after 31 days of culture (Chapter 3.1 – Figure 1). Constant motor neuron birth was even present when external trophic factor support was not present, leading to a considerable fraction of newborn human motor neurons over the course of 15 days (neurogenesis was also present beyond this 15-day period; Chapter 3.1 – Figure 1). This protracted duration of human motor neuron genesis in culture contrasts with the short <24-hour period of motor neuron production in differentiated mouse ES cell cultures (Peljto et al., 2010). These findings likely reflect in part

biological differences in the development of motor systems in rodent and human embryos, since human motor neurons are produced over an extended three-week period *in vivo* (Altman and Bayer, 2001; Bayer and Altman, 2002). Moreover, it remains to be determined whether during human spinal cord development the disappearance of motor neuron progenitors after the normal period of motor neurogenesis parallels what has been demonstrated in mouse (Mukouyama et al., 2006).

Interestingly, lower vertebrates display a capacity to continuously generate new motor neurons in response to injury throughout their life (Bhatt et al., 2004; Campbell et al., 2011; McHedlishvili et al., 2012; Monaghan et al., 2007; Reimer et al., 2009; Reimer et al., 2008; Seifert et al., 2012). These regenerative mechanisms have only recently started to be unravelled. For example, in the zebrafish a marked increase has been demonstrated in the proliferation of Olig2-positive ependymoradial glial progenitor cells in the ventricular zone following lesion of the spinal cord (Reimer et al., 2008). Subsequent studies showed that a subset of these progenitor cells maintain their capacity to produce large numbers of motor neurons into adulthood, a process which seems to be dependent on SHH signalling (Reimer et al., 2009) and inhibition of the NOTCH signalling (Dias et al., 2012). Those motor neurons become ChAT-positive cells nearly 6-8 weeks after lesion and seem to complete their full maturation and integration into the spinal circuitry (Reimer et al., 2008). In a similar manner, the Mexican salamander (also known as Axolotl) also displays the capacity to fully regenerate the spinal cord following lesion (Clarke et al., 1988; McHedlishvili et al., 2007). These amphibians have played an important role in the understanding of the regenerative abilities of the CNS and how it may be improved. The Axolotl is the only tetrapod known to fully functionally reconstitute a lesioned spinal cord and all the constituent tissues of the adult limb (McHedlishvili et al., 2012). The reconstitution of the repertoire of different cells present in the spinal cord can be accomplished through the amplification of single cells which display multipotency features typical of neural stem cells (Fei et al., 2014; McHedlishvili et al., 2012). The process is dependent on the presence of SOX2-positive neural stem cells, since the ablation of SOX2 expression after tail amputation halted neural stem cell proliferation and, thus, prevented spinal-cord specific regeneration (Fei et al., 2014). It is possible that comparable mechanisms might play a role in the ongoing motorneurongenesis observed in our hPSC motor neuron cultures (Chapter 3.1 – Figure 1).

The differences between human and mouse ESC-derived motor neuron cultures can also be linked to different culture conditions across both systems and/or the current inability to efficiently specify motor neurons from hPSCs, which leads to the generation from hPSCs of cellular populations constituted by a mixture of post-mitotic neurons and actively dividing neuronal progenitor cells, among other cell types (Boulting et al., 2011; Hu et al., 2010; Li et al., 2005; Nizzardo et al., 2010; Patani et al., 2011; Sareen et al., 2012; Takazawa et al., 2012). Uninterrupted motor neuron birth in hPSC-specified cultures may reflect a failure to to efficiently drive early progenitors towards a specific cell fate, since the dual SMAD signalling neuralization protocol used for most of this work led to a nearly 70-80% conversion of hPSCs into PAX6-positive neural progenitor cells (Roybon et al., 2013); but the final motor neuron yield at day 31 ranged from 5 until 45% (Chapters 3.1 and 3.2). Consequently, at the end of each differentiation period the incomplete conversion of hPSCs into spinal cord motor neurons yields other cell types, including actively dividing neuronal progenitors, which later give rise to new-born motor neuron populations. Interestingly, following our original findings, some collaborators (e.g. Kevin Eggan, personal communication) have investigated the ongoing motorneurogenesis in hPSC-derived motor neuron cultures and described lower levels of continuous motor neuron birth, which could be explained by different experimental conditions and quantification procedures across the two laboratories.

Ultimately, in a broader perspective the neurogenic phenomenon reported here imposes caution in the interpretation of studies involving the evaluation of human motor neuron numbers over time in experiments using mixed neuronal cultures specified in similar conditions. Many published studies, based on the experience with rodent systems, have assumed that the only human motor neurons present in the culture are the postmitotic cells that were seeded originally. This constitutes a major potential confound for some recently published studies describing conditions that affect “motor neuron survival”, as the addition of new neurons cannot be clearly dissociated from pure survival promoting effects (Di Giorgio et al., 2008; Ebert et al., 2009; Sareen et al., 2012). In 2008, Di Giorgio and colleagues studied the influence of glial cells carrying ALS mutations on the survival of human spinal motor neurons (Di Giorgio et al., 2008). They established co-culture assays by seeding non-purified human spinal ESC-derived motor neurons on top of primary murine glial cells overexpressing SOD1G93A or derived from non-transgenic mice (Di Giorgio et al., 2008). The

cultures were followed for several days, with the authors showing reduced number of Hb9-positive motor neurons at day 10 and day 20 post-dissociation when cultivated in the presence of SOD1G93A glia comparatively to wild-type glia (Di Giorgio et al., 2008). This led to the conclusion that glial cells overexpressing a specific ALS mutation were toxic to human spinal motor neurons (Di Giorgio et al., 2008). However, the data presented shows nearly similar levels of Hb9-positive motor neurons at day 10 and day 20 when cultured in the presence of SOD1G93A; and increased number of motor neurons at day 20 comparatively to day 10 when human motor neurons where grown in the presence of wild-type glia (Di Giorgio et al., 2008). Together, these results point to the possible presence of ongoing motorneurogenesis in this co-culture system and open the door to alternative explanations, including different rates of motor neuron formation in the presence or absence of apparently toxic glial cells (Di Giorgio et al., 2008). In line with this, the Svendsen lab has studied hiPSC-derived motor neurons generated from SMA patients and proposed a degenerative phenotype for those motor neurons having a SMA background (Ebert et al., 2009). However, in their studies non-purified human motor neuron cultures were used and thus the survival decreasing effect they report cannot formally be distinguished from an altered pattern of motor neuron genesis (Ebert et al., 2009).

Overall, the reported uninterrupted birth of human motor neurons in specified-hPSC cultures supports the need for a thorough characterization of the cellular types present in culture after submitting hPSCs to a differentiation protocol. Our data reinforce the need for more efficient differentiation protocols and more robust ways to isolate cells of interest from mixed neuronal/neural progenitor cultures (Allodi and Hedlund, 2014).

4.2. Screening for small molecules able to increase human motor neuron yields

In the past decade, numerous technological developments have made practical the idea of testing large collections of compounds on particular cellular populations under clearly defined and robust conditions (Carpenter, 2007a, b; Dragunow, 2008; Macarron et al., 2011; Mayr and Bojanic, 2009; Mitsumoto et al., 2006; Pepperkok and Ellenberg, 2006; Pruss, 2010). In this work we took advantage of these advances to further exploit the remarkably high level of continuous motor neuron birth in spinal cord-specified hPSC cultures. We followed an automated small-scale phenotypic screening approach to find compounds that display the capacity to increase the numbers of motor neurons in culture,

either by increasing neurogenesis or enhancing survival (Chapter 3.1 – Figure 2). We thought such molecules would be of value especially in light of the practical limitations in the yields of motor neurons specified from hPSCs. To facilitate the visualization of human motor neurons in culture, an assay was developed using mixed neuronal cultures specified from the reporter line HB9::GFP (HBG1 hESC line) (Di Giorgio et al., 2008). While conditions of neurotrophic deprivation were used as negative control, a cocktail of neurotrophic factors was used as positive control (Chapter 3.1 – Figure 2), taking into consideration their well-described pro-survival and pro-neurogenic effects during embryonic development (Gould and Enomoto, 2009; Gould and Oppenheim, 2011; Sendtner et al., 2000; Tovar et al., 2014). Using this assay we tested nearly 160 small molecules. The positive and negative controls chosen for this assay proved to be fairly consistent and reliable, because for each plate there was no overlap between the 6 positive and 6 negative control wells. This allowed running a first pass on a subset of the Microsource and Tocris collections, to check whether there were any human motor neuron relevant compounds. The only compound that was clearly above the rest was Y-27632 (Chapter 3.1 – Figure 2), a widely studied inhibitor of the Rho kinase (ROCK) (Ishizaki et al., 2000), whose activity was subsequently validated and is therefore not a false positive (discussed below). Thus, using this strategy we successfully identified a small molecule displaying robust capacity to enhance human motor neurons numbers in culture.

Interestingly, several compounds were shown to perform more poorly than the negative control and others were toxic for motor neurons and neuronal progenitors present in the cultures (Chapter 3.1 – Figure 2). However, these were not further explored. Even though the results obtained from this drug screening approach can be regarded as satisfactory and relevant, certain technical aspects of the methodology employed merit analysis and discussion. One aspect to discuss is the day 13 readout, which represents an unusually late time point for drug screening studies. Yet, during the optimization stage, the cultures were followed for 15 days and day 31+13 was demonstrated to be the time-point when the greatest differences in motor neuron numbers between positive and negative control conditions were registered. We reasoned that more active compounds would be captured if motor neuron counts could be performed at the point where the peak difference between control conditions was registered. Nevertheless, a late readout time-point raises concerns over drug stability in culture, and intrinsic variability of the readout; and it is therefore possible that some relevant hits could have been missed. If the assay could be

shortened it would be worth expanding this screening strategy to a larger compound collection. To increase the validity of the results obtained from the small-scale screen, we chose to test drugs in quadruplicate, using four sister testing plates. Our data demonstrated that controls and the great majority of compounds behaved consistently across the different plates. However, the use of so many replicates would not be economically feasible for larger screens, meaning that it would be important to further optimize the Z-score of the assay (Zhang et al., 1999). Taken together, we took advantage of the ongoing motor neurogenesis and successfully set up a low-throughput screening strategy which allowed us to identify the ROCK inhibitor Y-27632 as a relevant compound to help culturing hPSC-derived motor neuron cultures (Chapter 3.1 – Figure 2).

4.3. The hit compound Y-27632 increases motor neuron numbers in hPSC-derived cultures through enhancement of progenitor proliferation and motor neuron survival

The small-scale screening approach we developed following the observation of ongoing motor neuron birth in day 31+ hPSC-cultures allowed the identification of Y-27632 as a compound capable of robustly increasing the number of human motor neurons in culture. This molecule was originally described as an inhibitor of the Rho-associated kinase (ROCK) (Ishizaki et al., 2000), although subsequent studies showed that above a certain concentration it can also inhibit other kinases with similar potency (discussed below) (Davies et al., 2000; Nichols et al., 2009). ROCK belongs to a family of serine/threonine kinases, has a molecular mass of about 160 kDa and is one of the main downstream effector arms of the Rho pathway, namely the small GTPase RhoA (Riento and Ridley, 2003). Two isoforms of ROCK have been characterized so far: ROCK1 (also known as ROKβ) and ROCK2 (also known as ROKα) (Julian and Olson, 2014; Nakagawa et al., 1996; Pearce et al., 2010; Riento and Ridley, 2003). The ROCK1 gene is located on chromosome 18 and encodes a 1354-amino acid protein, whose expression is ubiquitous (Ishizaki et al., 1996; Matsui et al., 1996). The ROCK2 gene is located on chromosome 2 and encodes a 1388-amino acid polypeptide mainly expressed in the brain, muscle and heart (Matsui et al., 1996; Nakagawa et al., 1996). In terms of ultrastructure, ROCK1 and ROCK2 have an N-terminal kinase domain, a central coiled-coil-forming region containing a Rho-binding domain (RBD) and a pleckstrin homology (PH) motif with a C-terminal cysteine-rich domain (CRD) (Julian and Olson, 2014; Olson, 2008; Riento and Ridley, 2003). In the resting state, both ROCK1 and ROCK2 are essentially

cytosolic, but they are rapidly translocated to the membrane upon Rho activation (Olson, 2008; Riento and Ridley, 2003). ROCK has an auto-inhibitory activity, since in the inactive form its carboxyl terminal PH domain and RBD interact with the kinase domain, forming an auto-inhibitory loop (Olson, 2008; Riento and Ridley, 2003). This interaction is disrupted by binding of active Rho and thus kinase activity is increased (Amano et al., 1999; Julian and Olson, 2014). The two isoforms of ROCK have a global homology of 65% in their amino acid sequences and 92% homology in their kinase domains (Riento and Ridley, 2003; Tonges et al., 2011). ROCKs are also homologous to other members of the AGC kinase family, such as myotonic dystrophy kinase-related CDC42-binding kinase (MRCK), myotonic dystrophy kinase (DMPK) and citron kinase (Pearce et al., 2010). The Rho-ROCK pathway is a conserved cellular pathway that plays vital roles in diverse aspects of cell behaviour, including motility, cell division, apoptosis and neurite outgrowth, among other processes (Coleman and Olson, 2002; Coque et al., 2014; Etienne-Manneville and Hall, 2002; Hall and Lalli, 2010; Ohgushi and Sasai, 2011; Olson, 2008; Riento and Ridley, 2003; Vega and Ridley, 2008). After detailed characterization, we could demonstrate that Y-27632 increased motor neuron numbers not only by boosting progenitor proliferation in hPSC-specified motor neuron cultures, but also by promoting the survival of those motor neurons present in culture (Chapter 3.1 – Figures 2, 3 and 5). However, we were not able to determine whether Y-27632 could also exert any influence on the survival of progenitors in motor neuron lineage.

Regarding the pro-proliferative properties of Y-27632 in these cultures (Chapter 3.1 – Figures 2, 3 and 5), it is interesting to learn from the current literature that this small molecule is paradoxically linked to both pro-proliferative and anti-proliferative effects. On one hand, Y-27632 is a strong promoter of the survival and proliferation of hESCs and hiPSCs (Chen et al., 2010; Ohgushi et al., 2010; Ohgushi and Sasai, 2011; Watanabe et al., 2007), the immortalization and proliferation of keratinocytes (Chapman et al., 2010; Chapman et al., 2014; McMullan et al., 2003; van den Bogaard et al., 2012), proliferation of hPSC-derived retinal pigmental epithelium (Croze et al., 2014), proliferation of murine astrocytes (Yu et al., 2012), proliferation of human neuroblastoma cells (Street et al., 2010) and adipogenesis (Noguchi et al., 2007). For example, in the particular case of hPSC-derived retinal pigmental epithelium, Y-27632 was shown to significantly alter gene expression towards a profile associated with enhanced cell cycle progression (Croze et al., 2014). Among the different genes altered, there was an upregulation of Cyclin-dependent kinase 1 (CDK-1), Proliferating

cell nuclear antigen (PCNA) and Cyclin A2 (CCNA2); with concomitant decrease in Cyclin-dependent kinase inhibitor 2B (CDKN2B) (Croze et al., 2014). In human neuroblastoma cells, Y-27632 treatment was shown to induce a more rapid progression through the cell cycle and, therefore, enhanced proliferative rates (Street et al., 2010). It is plausible that similar mechanisms could play a role in the Y-27632-induced increase in proliferation observed in our hPSC-derived motor neuron cultures, which resulted in increased motor neuron numbers at day 31+9 (Chapter 3.1 – Figures 2 and 3).

On the other hand, it has been reported that Y-27632 is also able to induce strong anti-proliferative effects in various types of cancer cells (Burthem et al., 2007; Routhier et al., 2010; Zohrabian et al., 2009), cord blood-derived $CD34^+$ hematopoietic progenitor cells (Bueno et al., 2010), hepatic stellate cells (Iwamoto et al., 2000), cardiac myocytes (Zhao and Rivkees, 2003) and smooth muscle cells (Rees et al., 2003; Sawada et al., 2000). In line with this, we extended the observation of anti-proliferative effects for Y-27632 by showing in this Thesis the non-benefitial use of Y-27632 for the expansion of human adipose-derived stem cells (hADSCs; Chapter 3.5; discussed below). These differences in the proliferative effects of Y-27632 might be related to cell type-specific profiles or depend on the maturational stage of the cells. In our hESC-motor neuron system, the pro-proliferative effects of Y-27632 seem to promote a “pro-motorneurogenic profile” since supplementation of spinal hPSC cultures with Y-27632 led to remarkable increases in the numbers not only of motor neurons, but also Olig2-positive progenitors. These effects were detected in both hESC- and hiPSC-derived cultures, strengthening the general relevance of our findings (Chapter 3.1 – Figure 3). However, the effects induced by Y-27632 in the hPSC-derived motor neuron cultures most likely are not motor neuron restricted and, therefore, it would be interesting to test the effect of the compound in other cultures specified from hPSCs, including dopaminergic neurons (Kriks et al., 2011), cardiomyocytes (Dambrot et al., 2011; Davis et al., 2012) or pancreatic β-cells (Pagliuca et al., 2014). The increase in motor neuron progenitor numbers induced by Y-27632 could also be related to interactions with pathways implicated in the regular maintenance of neuronal progenitors or in triggering particular CNS regenerative mechanisms similar to the ones already described for lower vertebrates in response to injury (McHedlishvili et al., 2012; Monaghan et al., 2007). It also remains to be established whether the addition of Y-27632 to mixed motor neuron cultures induces changes in other non-spinal motor neuron and non-motor neuron progenitor cell types present in the cellular mixture.

Indeed, it is possible that Y-27632 causes other cells in the microenvironment to secrete factors that later act on motor neuron progenitors or motor neurons, leading ultimately to the effects we observed during our study. Even though we have not fully characterized the hPSC-cultures in this work, human astrocytes are likely to be present in culture in limited amounts (Dimos et al., 2008; Li et al., 2005) and we have recently shown that that hPSC-derived astrocytes express and secret both BDNF and GDNF (Roybon et al., 2013). Interestingly, in primary murine astrocyte cultures the presence of Y-27632 induces the expression of the pro-neurogenic and pro-survival factor BDNF, among other pro-survival molecules (Lau et al., 2012). Future studies should address this possibility more thoroughly.

The purification of human motor neurons using FACS technology allowed us to exclude the indirect effects on motor neurons of non-motor neuron cells present in hPSC-derived motor neuron cultures (Chapter 3.1). Using this approach we demonstrated that Y-27632 not only promotes human motor neuron axonal outgrowth (Chapter 3.3), but also increases the survival of human motor neurons cultured in the absence of neurotrophic factors for 7 days (Chapter 3.1 – Figure 6). While motor axon outgrowth enhancement in the presence of Y-27632 was an expected result based in several previous reports (Bito et al., 2000; Dergham et al., 2002; Duffy et al., 2009; Fournier et al., 2003; Monnier et al., 2003; Watzlawick et al., 2014), the survival promoting effect of Y-27632 is a novel finding with potential clinical implications. Even though Y-27632 was not able to rescue patient-specific hiPSC-derived dopaminergic neurons from different oxidative insults (Nguyen et al., 2011), increasing evidence proposes Y-27632 as a robust neuroprotective agent for different types of neuronal populations (Gisselsson et al., 2010; James et al., 2008; Jeon et al., 2012; Julien et al., 2008), including spinal motor neurons. A recent report demonstrated that Y-27632 is capable of protecting murine hippocampal neurons *in vitro* from glutamate-induced excitotoxicity and *in vivo* from kainic acid-induced neurodegeneration (Jeon et al., 2012, 2013), which adds to a previous report showing neuroprotective effects of Y-27632 in CA1 hippocampal cells in an *in vitro* cerebral ischemia model employing organotypic hippocampal slice cultures (Gisselsson et al., 2010). Cerebellar Purkinje cells (Julien et al., 2008) and retinal ganglion cells (Lingor et al., 2008; Yang et al., 2009) are also among neuronal cell types shown to be protected by Y-27632 in injury contexts. In addition, Y-27632 has been explored as a strategy against chemotherapy-induced neuropathy, displaying promising neuroprotective effects (James et al., 2008; James et al., 2010).

In terms of spinal motor neurons, Y-27632 was shown to increase the survival and promote the neurite outgrowth of primary motor neurons *in vitro*, but failed to extend the survival of SOD1(G93A) mice (Gunther et al., 2014). Other recent studies documented an increase in the lifespan of an intermediate mouse model of SMA when mice were given Y-27632 for a prolonged time (Bowerman et al., 2010). The compound was shown not to be capable to halt motor neuron loss in the ventral horn of the spinal cord, which is not a key feature of this model; but showed positive effects on the maturation of the neuromuscular junction (NMJ) and muscle fiber size (Bowerman et al., 2010). Interestingly, Fasudil, another small molecule in the ROCK inhibitor family, was also shown to slow disease progression, increase survival time and reduce motor neuron loss in a SOD1G93A mouse model of ALS; and improve the survival of SMA mice, not by halting motor neuron loss, but by inducing beneficial effects on the muscle with preservation of the neuromuscular junction. In the particular case of SMA mice the effect on survival was more pronounced with Fasudil than with Y-27632 (Bowerman et al., 2010; Bowerman et al., 2012). In our study, Fasudil was able to increase axonal outgrowth, but could not increase the survival of neurotrophically-deprived hESC-motor neurons (Chapter 3.3; discussed below).

In terms of molecular mechanisms, blockade of the Rho-ROCK pathway is a molecular approach demonstrated to enhance the neurite outgrowth of spinal motor neurons (Dergham et al., 2002; Forgione and Fehlings, 2014; Fournier et al., 2003; Watzlawick et al., 2014; Wu et al., 2009). The activation of the Rho-ROCK pathway leads to the collapse of the growth cone and neurite retraction, not only by inducing microtubule destabilization through activation of collapsin response mediator protein 2 (CRMP2) (Watzlawick et al., 2014); but also by interfering with the actin cytoskeleton through activation of myosin light chain (MLC) and LIM kinase (LIMK), which in addition causes cell contraction and stress fiber formation (Tonges et al., 2011; Watzlawick et al., 2014). Another potential target of ROCK activation is phosphatase and tensin homologue (PTEN), whose activity has been linked to axonal outgrowth abrogation (Park et al., 2008). The blockade of these pathways counteracts the Rho-ROCK-induced inhibition of axonal regeneration following spinal cord nerve injury (Watzlawick et al., 2014).

While the mechanisms underlying increased axonal outgrowth upon ROCK inhibition are in part established, the pathways accounting for the survival inducing effect of Y-27632 remain elusive. Increased ROCK activation is linked to the activation of cell death

pathways and to the cellular events leading to apoptosis, including cell contraction, membrane blebbing, nuclear disentanglement and the final fragmentation of apoptotic cells into apoptotic bodies (Coleman and Olson, 2002; Shi and Wei, 2007). These mechanisms involve the activation of MLC phosphorylation, and actomyosin contraction and seem to be caspase-3 dependent (Coleman and Olson, 2002; Julian and Olson, 2014). Thus, Y-27632 could be contributing to enhanced human motor neuron survival by disrupting some of these mechanisms, similarly to its ability to halt the hESC dissociation-induced apoptosis (Chen et al., 2010; Ohgushi et al., 2010; Watanabe et al., 2007). However, it remains an open question whether Y-27632 is able to induce the survival of hESC-purified motor neuron in a ROCK-dependent manner (discussed below). Some pioneer studies have demonstrated beneficial effects upon combining Y-27632 with other neurotrophic factors and small molecules (Ahmed et al., 2009; Bermel et al., 2009; Lingor et al., 2008). One of those studies showed the synergistic actions of Y-27632 combined with CNTF to enhance survival and regeneration of retinal ganglion cells under *in vitro* injury paradigms (Lingor et al., 2008). It would be interesting to pursue similar studies involving Y-27632 and other neurotrophic factors in hPSC-derived motor neuron cultures. Taken together, the data reported here add further evidence for the neuronal protective roles of Y-27632.

4.4. Y-27632 increases human motor neuron numbers likely through a ROCK-independent mechanism

Among the eight different small molecules belonging to the ROCK Inhibitor family tested (Fasudil, HA-1100, H-1152, GSK429286, SR3677, Thiazovivin, Y-27632 and Y-39983) the only compound capable of significantly increasing the number of human motor neurons in culture was Y-27632 (mixed survival/proliferation assay and FACS-purified motor neuron assay; Chapter 3.3). These results raised questions about the molecular mechanisms underlying the effects we observed, especially because Y-39983 is a newer and allegedly improved version of Y-27632 and has been linked to a more effective inhibition of the Rho-ROCK pathway (Tokushige et al., 2007). To address this question further we set up a phenotypic 48-hour neurite outgrowth assay given that inhibition of the Rho-ROCK pathway has been unequivocally recognized to stimulate neurite elongation in motor axons (Bito et al., 2000; Dergham et al., 2002; Fournier et al., 2003; Monnier et al., 2003; Wu et al., 2009). Our data showed that all tested ROCK inhibitor molecules stimulated *in vitro* axonal

outgrowth by motor neurons, in agreement with the data from the literature demonstrating that all eight molecules inhibit the Rho-ROCK pathway at the concentrations tested (Chapter 3.3). Our results raise the possibility that the increase in human motor neuron numbers induced by Y-27632 is not predominantly linked to the inhibition of the Rho-ROCK pathway. A similar conclusion may be extended to the present discovery that Y-27632 is a motor neuron survival factor. The observations of Kobayashi and colleagues are striking in this context (Kobayashi et al., 2004). They created transgenic mice expressing a dominant-negative form of ROCK to study the role of the Rho-ROCK pathway in the embryonic development of motor neurons in the spinal cord (Kobayashi et al., 2004). They observed enhanced motor neuron death both during early and late embryonic stages (Kobayashi et al., 2004). This suggested that the Rho-ROCK signaling pathway plays a critical role in the survival of spinal motor neurons during embryonic development (Kobayashi et al., 2004), which is in clear contrast with our observation of the survival promoting effects induced by Y-27632 on human embryonic motor neurons. Therefore, it is possible that unknown cellular targets or other kinases inhibited by Y-27632 in this concentration range may be involved. Two groups have tested *in vitro* Y-27632 and other protein kinase inhibitors against large panels of protein kinases and showed that at 10 µM, besides ROCK, the compound Y-27632 can robustly inhibit other kinases, namely protein kinase C-related kinase 2 (PRK2), mitogen- and stress-activated protein kinase-1 (MSK1), leucine-rich repeat protein kinase-2 (LRRK2) and MAP kinase interacting kinase 1 (MNK1) (Davies et al., 2000; Nichols et al., 2009). This suggests a third scenario, in which the observed effects of Y-27632 could be linked to the concomitant inhibition of several kinases in an exclusive combination. Studies using P8 mouse cerebellar neurons seem to support this interpretation, since the Y-27632 molecule alone elicited a more powerful stimulation of neurite elongation than the siRNA knockdown of ROCK1 and ROCK2 alone, PRK2 alone or ROCK1 and ROCK2 in parallel (Darenfed et al., 2007). Altogether, our results add to the current literature and reinforce the need for a more thorough understanding of the molecular pathways affected by Y-27632. If one could unravel the detailed plethora of actions of Y-27632 on hPSC-derived motor neurons and their progenitor populations we could possibly find ways to more rigorously drive motor neuron amplification *in vitro.*

4.5. Translating Y-27632 into the clinic?

The Rho kinase inhibitors are a class of small molecules displaying pleiotropic and wide-ranging beneficial effects across different cell types. As a result, these drugs have been explored as therapeutic agents for a panoply of pathologic conditions, including hypertensive vascular disease (Antoniu, 2012; Hirooka et al., 2004; Lohn et al., 2009; Nishikimi et al., 2007; Satoh et al., 2011), stroke (Li et al., 2009; Rikitake et al., 2005; Satoh et al., 2007; Shibuya et al., 2005; Yano et al., 2008), heart failure (Fukui et al., 2008; Satoh et al., 2011; Wang et al., 2011), cerebral vasospasm associated with subarachnoid haemorrhage (Liu et al., 2012; Satoh et al., 2012; Velat et al., 2011; Zhao et al., 2006; Zhao et al., 2011), glaucoma (Fukunaga et al., 2009; Waki et al., 2001; Watabe et al., 2011), different forms of cancer (Deng et al., 2010; Itoh et al., 1999; Takamura et al., 2001; Takeba et al., 2012), erectile dysfunction (Chitaley et al., 2001; Guagnini et al., 2012; Saito et al., 2012) or diabetes mellitus (Hammar et al., 2009; Komers, 2011), multiple sclerosis (Sun et al., 2006), Parkinson's disease (Borrajo et al., 2014; Tonges et al., 2012), ALS (Gunther et al., 2014; Takata et al., 2013), SMA (Bowerman et al., 2012; Coque et al., 2014), among others. One of the compounds belonging to this family is Fasudil, which has been in clinical use in Japan since 1995 with promising results in the prevention of the vasospasm associated with subarachnoid hemorrhage (Zhao et al., 2006; Zhao et al., 2011). Fasudil was demonstrated to be at least as effective as the gold-standard treatment using the putative L-type voltage-gated calcium channel blocker agent nimodipine (Zhao et al., 2006; Zhao et al., 2011). Follow-up surveillance studies have revealed that Fasudil was well tolerated and safe in patients with subarachnoid hemorrhage (Zhao et al., 2006; Zhao et al., 2011). Recent clinical trials have evaluated whether Fasudil might be a useful therapeutic option for Reynaud's phenomenon (ClinicalTrials.gov Identifier: NCT00498615) or improvement of vascular function (ClinicalTrials.gov Identifier: NCT00120718, NCT00670202 and NCT01069042). In other clinical trials researchers have studied the novel ROCK Inhibitor SAR407899 for Erectile Dysfunction (ClinicalTrials.gov Identifier: NCT00914277) and Chronic Kidney Disease (ClinicalTrials.gov Identifier: NCT01485900). Together, these pioneer attempts to use this class of compounds clinically support the idea they are possibly safe and can be well tolerated by patients. Interestingly, a phase 1 clinical trial of safety and efficacy of Fasudil in subjects with ALS (ClinicalTrials.gov Identifier: NCT01935518) is actively recruiting patients. ALS patients involved in the trial will take Fasudil treatment for 14 days (30 mg twice a day,

intravenous); and 3 months later will repeat the same treatment. All the patients will be followed up for 6 months and the primary outcome will be the decline rate of ALSFRS-R. It remains to be determined whether Y-27632 has a similar safety profile, since it has not been tested clinically. The present data suggest that Y-27632 merits a more in-depth pre-clinical assessment as a possible therapeutic option in patients suffering from ALS or other forms of motor neuron disease. Rationale for this is provided by our observation that Y-27632 is capable of promoting the survival of human motor neurons deprived of neurotrophic factors for nearly one week and also some previous work using animal models. The already described effects of Y-27632 on motor neurons could help to pave the way for clinical trials in human patients. However, before one move to this stage comprehensive pre-clinical data on the molecular mechanisms, safety and efficacy of the strategy should be acquired. In addition, there will be a prerequisite to establish effective, minimally invasive and standardized approaches for adequate drug delivery. One concern is that the penetration of Y-27632 in the CNS is too low to achieve therapeutic levels and studies on this topic are scarce. Interestingly, when used in an *in vitro* model of blood-brain barrier (BBB), Y-27632 helped to improve the integrity of the BBB (Allen et al., 2010; Persidsky et al., 2006).

Together, we exploited ongoing generation of motor neurons in hPSC-specified cultures and found Y-27632, a compound that might open new avenues to the study of human motor neurons, not only at the bench, but also possibly at the bedside.

4.6. A new robust survival assay using purified human motor neurons opens novel avenues for meaningful drug discovery

The continuous generation of motor neurons in hPSC-derived motor neuron cultures described here raises fundamental concerns regarding the interpretation of studies relying solely on total cell counts to evaluate neuronal survival over time. We reasoned that the most accurate method to study motor neuron survival would be to isolate these cells from the non-motor neuron fraction present in the mixed cultures (Chapter 3.1 – Figure 4). Our initial attempts involved the direct usage of mitotic inhibitors to halt ongoing proliferation in mixed cultures. In our hands, the strategy was demonstrated not to be feasible since the compounds would induce toxic effects in the mixed cultures: cytosine arabinoside (AraC) was shown to be toxic to human motor neurons, while the less toxic uridine/fluorodeoxyuridine (U/FDU) led to clumping of neuronal cells on remaining islands of

dying proliferative cells (not shown). Therefore, we took advantage of the HB9::GFP reporter line (Di Giorgio et al., 2008) and used FACS to purify the GFP-positive motor neurons (Placantonakis et al., 2009; Singh Roy et al., 2005). In each experiment, the pool of cells utilized was maximized beforehand through amplification of motor neuron numbers using Y-27632 supplementation for nearly 3 days (Chapter 3.1 – Figure 4). Using this approach we could develop a robust 7-day motor neuron survival assay and for the first time show that well-established neurotrophic factors (GDNF, BDNF and CNTF; Chapter 3.1 – Figure 5), similarly to rodent and chick neurons, can enhance the survival of human motor neurons (Gould and Enomoto, 2009; Gould and Oppenheim, 2011; Henderson et al., 1998; Kanning et al., 2010). Also, in line with previous studies we could demonstrate that supplementation of the cultures with compounds like Forskolin and IBMX (Hanson et al., 1998; Montoya et al., 2009), which induce an increase in the endogenous levels of cAMP, could strongly potentiate the effect of all neurotrophic factors tested [significant effects for GDNF and IGF-1 (not shown)] and improve the survival of human motor neurons Chapter 3.1 – Figure 5). In addition, using this novel survival assay we could demonstrate a motor neuron survival-promoting effect for Y-27632 Chapter 3.1 – Figure 6). Only by adopting a purification strategy can the confounding effects of ongoing neurogenesis and other indirect signalling mechanisms be overcome. Indeed, historically, the discovery of motor neuron survival factors was a slow process and the first discoveries were only made once purified preparations of primary motor neurons became available. To our knowledge this is the first motor neuron survival assay utilizing an almost pure population of human motor neurons.

During the preparation of this Thesis, other groups published human motor neuron survival assays based on purified human motor neurons (Egawa et al., 2012; Yang et al., 2013). One survival assay that has captured the attention of the scientific community was developed by the Rubin group at Harvard University (Yang et al., 2013). They used FACS-purified motor neurons to conduct survival experiments, however those experiments required a mouse astrocyte monolayer as substrate and blockage of cell proliferation with AraC (Yang et al., 2013), which was demonstrated to be cytotoxic in our hands. The purified hPSC-derived motor neurons were cultivated for nearly 20 days after purification: the first 6 days supplemented with a cocktail of neurotrophic factors (20 ng/mL BDNF, GDNF and CNTF) and the remaining 14 days in the absence of neurotrophic factor supplementation (Yang et al., 2013). This assay led to the discovery of kenpaullone, a dual inhibitor of GSK-3 and HGK

kinases, which was shown to promote the survival of both hESC-derived motor neurons and patient-specific hiPSC-derived motor neurons harbouring SOD1 mutations (Yang et al., 2013). However, the effects of the drug on the astrocyte monolayer could not be isolated, in contrast to the survival assay developed in this Thesis (Chapter 3.1 – Figure 4). Nevertheless, the work by the Rubin group represented one of the first successful attempts to use human pluripotent stem cells to find candidate drugs to treat motor neuron disorders (Yang et al., 2013). In addition, their approach is a remarkable example of future "preclinical testing in the dish", since they tested other candidate drugs like dexapramipexole, which showed promising results in mouse models, but failed to increase the survival of human motor neurons carrying SOD1 mutations in their study (Yang et al., 2013).

One important challenge for *in vitro* studies involving hPSC-derived motor neurons is to characterize the maturity of the cells used in the assays. It is anticipated that the generation of human cells which more closely resemble the cell type of interest will possibly yield data which is more relevant from the biological point of view. The dependence on neurotrophic factors is acquired over time during embryogenesis (Mettling et al., 1995). The amplified and purified human motor neurons we used in this work were responsive to neurotrophic factors and, therefore the stage of maturation of those motor neurons is comparable to mid-embryonic period in mice. Therefore, novel strategies to efficiently enhance the maturation of hPSC-motor neurons are needed to generate human motor neurons which are alike adult motor neurons. Nevertheless, we anticipate that the survival assay we developed during this Thesis based on purified human motor neurons might soon constitute a powerful tool for drug screens to identify compounds which are neuroprotective for human motor neurons.

In this regard, a parallel can be established with the experience using rodent and chick primary motor neuron cultures during the 1990's. Nearly two decades ago in a rather similar approach motor neuron researchers used purified chick and rodent primary motor neurons to identify a myriad of neurotrophic factors which could keep embryonic motor neurons alive and which were later shown to have protective roles in animal models of the motor neurodegenerative disease ALS (Bar, 2000; Beck et al., 2001; Gouin et al., 1993; Henderson et al., 1993a; Henderson et al., 1993b; Henderson et al., 1993c; Henderson et al., 1994; Pennica et al., 1996; Sendtner et al., 2000). To add further to the relevance of the human motor neuron survival assay we developed here, a few years ago the French-based

biopharmaceutical company Trophos using primary rodent motor neurons developed a similar assay and tested tens of thousands of small molecules to find neuroprotective compounds for motor neurons (Bordet et al., 2007). TRO19622 (Olexosime) was the most promising small molecule identified in that large-scale screen (Bordet et al., 2007). The preclinical studies showed that through interactions with the mitochondrial permeability transition pore (mPTP) the compound was capable of eliciting robust survival effects in neuronal populations under injury conditions (Bordet et al., 2007; Martin, 2010). The drug was further developed and clinical trials were conducted recently in Europe for ALS and SMA (ClinicalTrials.gov Identifier: NCT00868166 and NCT01285583 for ALS; NCT01302600 for SMA). In a phase III clinical trial for ALS the compound failed to demonstrate a significant increase in survival versus placebo (ClinicalTrials.gov Identifier: NCT00868166 and NCT01285583). However, this year the company announced promising results regarding the possible use of Olexosime as a therapeutic strategy in SMA patients. The data gathered from the phase II clinical trial conducted for type II and type III SMA patients (ClinicalTrials.gov Identifier: NCT01302600) demonstrated an enhanced preservation of motor function upon Olexosime treatment, combined with fewer adverse events when compared to placebo. Together, these results have raised the hope that Olexosime could soon become the first ever available treatment developed specifically for SMA patients. We therefore succeeded in developing a strategy and an assay based on an almost pure population of motor neurons which allow us not only to study survival of human motor neurons, but also to possibly test thousands of compounds under optimized conditions.

4.7. Purification of hPSC-motor neurons allows the development of robust assays to study pathological and non-pathological conditions involving human motor neurons

The strategy developed in this work to maximize motor neuron yields from hPSC-derived motor neuron cultures coupled with the capacity to safely purify human motor neurons to create robust conditions to develop reproducible assays has opened far-reaching possibilities regarding the study of conditions which affect motor neurons. In collaborative experiments performed with other colleagues in the laboratory we were able to demonstrate the marked axonal outgrowth promoting capacities of statins to human motor neurons (Chapter 3.4), even in the presence of well-known inhibitory substrates for regeneration (myelin and chondroitin sulfate proteoglycans) (Domeniconi and Filbin, 2005;

Filbin, 1995; Gopalakrishnan et al., 2008; Grados-Munro and Fournier, 2003; Monnier et al., 2003; Morgenstern et al., 2002; Nash et al., 2009). These human studies followed the initial identification of simvastatin as the most robust hit compound from a 50,000 small molecule screen performed using mESC-motor neurons to find new compounds that promote motor axon elongation (Chapter 3.4).

Epidemiological studies have proposed the organophosphate pesticides as motor neuron death triggering agents in patients (Kamel et al., 2012; Malek et al., 2012; McGuire et al., 1997; Saeed et al., 2006; Slowik et al., 2006) and the survival assay we developed here has allowed the evaluation of this association *in vitro* using human motor neurons derived from hPSCs (Prissette, M. *et al.*, in preparation). We took advantage of the robust assay we developed in the current work to study the direct and indirect toxic effects of pesticides in an almost pure population of human motor neurons.

Finally, we were able to further amplify the usage of the assay and develop a robust co-culture platform with hPSC-astrocytes, which we predict will translate into insights regarding the role of motor neuron-astrocyte interactions in physiologic and disease contexts (Roybon et al., 2013).

Thus, the purification strategy for human motor neurons and the assay developed during this work has allowed not only studies on the survival of human motor neurons, but also to perform a panoply of other relevant experiments which we hope will lead to a better understanding of physiologic/disease contexts involving human motor neurons and also opens the prospect of identifying drugs with real clinical impact on motor neuron disease.

On the disease modelling perspective, if better strategies of motor neuron purification could be coupled with the proposed Y-27632 expansion of hiPSC-motor neuron cultures, larger pools of human motor neurons from healthy and diseased donors could be studied. The possibility of using hiPSC-derived cells to compare healthy controls and patient specific-purified motor neurons will possibly lead to the establishment of robust *in vitro* disease-related phenotypes (for example, altered survival, shorter neurites, increased protein aggregation), which can easily be explored in robust assays to be assessed in unbiased, high-throughput drug screening campaigns. However, it remains to be determined if the pro-neurogenic and pro-survival capacities displayed by Y-27632 can possibly mask *in vitro* disease phenotypes in cultures derived from patients with motor neuron disease. In the case of motor neurodegenerative disorders, the relevant molecular hits resulting from here

will possibly be more clinically meaningful, because they are tested in human cells; which could result into a more rapid translation into effective therapeutic strategies. It is important to highlight that some of the compounds available in the libraries are already approved by FDA and EMA for human use, opening the prospect for a more rapid translation into an effective strategy in case the *in vitro* data is shown to be promising. In addition, the access to large quantities of purified motor neurons from controls and patients allow the investigation of disease mechanisms which cannot be revealed by studying postmortem samples, including cellular events that maybe take place before overt disease is evident.

Ultimately, it is possible that the body of work generated using these purified motor neurons can soon be translated to other neuronal populations and to the study of other neurological disorders.

4.8. Human ADSCs are potentially an alternative source of patient-specific stem cells, whose cultivation is not enhanced upon exposure to Y-27632

Human ADSCs have been proposed in the past decade as an accessible potential source of personalized stem cells, especially because these stem cells are simple to isolate and to grow *in vitro* (Gimble and Guilak, 2003). Here, in order to better understand the mechanisms that regulate their survival and proliferation, we studied the effect of Y-27632 on the cultivation of those multipotent stem cells (Chapter 3.5). Unlike the pro-proliferative and pro-survival effects described for hPSC-derived motor neuron cultures and other stem cell types (discussed above), the supplementation of hADSCs cultures with Y-27632 was shown not to increase the plating efficiency of hADSCs and also failed to enhance the expansion of this type of human adult stem cells (Chapter 3.5). Interestingly, we were able to propose a novel stem cell type in which Y-27632 supplementation does not constitute a successful strategy to improve its culture. It will be interesting to further unravel thoroughly the cellular mechanisms accounting for the heterogeneous behavior of different stem cell types upon Y-27632 treatment. In light of the new knowledge on how to efficiently commit hPSCs into neural tissue, one can anticipate that parallel strategies can possibly be explored to drive hADSCs towards a neural fate. Ultimately, these fat-derived stem cells can constitute a reliable novel source of personalized human motor neurons.

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CHAPTER 5

CONCLUSION

OUTSANDING QUESTIONS

AND FUTURE PERSPECTIVES

5.1. CONCLUSION

Human pluripotent stem cells (hPSCs) have opened new paths of innovation and enthusiasm in developmental biology, drug discovery and disease modelling. However, much work remains to be done before we can exploit the complete potential of these unique cells. Here, we showed that Y-27632 can be utilized to amplify the ongoing birth of motor neurons in hPSC cultures and considerably increase the production of human spinal cord motor neurons specified from hPSCs. Combining the Y-27632-induced increase in motor neuron numbers with FACS purification we could develop a robust assay to test survival promoting molecules in a refined population of human motor neurons. It is our hope that the work reported in this thesis will lead to reliable and robust human motor neuron assays to evaluate survival and toxicity, electrophysiological studies and analysis of disease phenotypes using patient-specific hiPSC-derived motor neurons.

Contrarily, in hADSCs, which constitute a possible new source of personalized stem cells, the supplementation of cultures with Y-27632 was not linked to a pro-survival and pro-proliferation effect. Accordingly, Y-27632 was demonstrated to be detrimental to the culture of these multipotent stem cells and, thus, we could propose hADSCs as a cell type uncommonly negatively affected by the presence of Y-27632.

Finally, the methods developed in the current work and reported here should be of global interest and general application for everyone using human stem cells to study healthy and disease-specific cell types.

In brief, the novel aspects reported here are:

> Ongoing motor neuron birth in spinal cord cultures specified from hESCs/hiPSCs.

> The continuous generation of motor neurons is a potential confound for motor neuron survival studies using mixed post-mitotic/neuronal progenitor cultures.

> Y-27632 increases the yield of human motor neurons from hESCs and hiPSCs lines, probably through the enhanced stimulation of proliferation of motor neuron progenitors.

> Y-27632 is likely to enhance motor neuron generation through a ROCK-independent mechanism.

> Y-27632 promotes the survival and neurite outgrowth of purified hESC-motor neurons.

> Human motor neurons amplified with Y-27632 can be purified by FACS and used in reproducible survival assays to reveal a marked response to well-known neurotrophic factors.

> The survival and proliferation of the multipotent human ADSCs is unexpectedly not enhanced upon Y-27632 supplementation.

5.2. OUTSTANDING QUESTIONS AND FUTURE PERSPECTIVES

In addition to the findings summarized above this thesis opened novel research avenues and raised many outstanding questions worth thoroughly pursuing in future studies. Some that merit a comprehensive evaluation are:

1. We documented continuous motor neuron birth in dissociated Day 31+ hPSC-derived motor neuron cultures. Is this a consequence of the extended period of neurogenesis in humans? Or is this a consequence of the current inability of protocols to efficiently specify a pure motor neuron population and/or mature cell types from hPSCs? Or does it reflect an unintentional aspect of the specific culture conditions used? Longer periods of motor neuron differentiation from hPSCs will possibly help addressing some of these questions. On the other hand, we could aim to conduct successive passaging experiments and assess whether we can exhaust progenitor populations in the cultures differentiated from hPSCs. Another interesting and fundamental experiment would be the purification of progenitors from mouse and human to compare their intrinsic properties in identical conditions.

2. The 160-compound screening approach employed here using mixed cultures led to the discovery of Y-27632, which displayed a marked capacity to increase human motor neuron numbers in culture. If it were possible to devise a shorter, more robust assay, it would be interesting to increase the number and range of compounds to screen in single wells, so that we could possibly find other small molecules displaying relevant characteristics in hPSC-derived motor neuron cultures.

3. We have demonstrated that the number of Olig2-positive progenitor cells increases over time in the presence of Y-27632. Does Y-27632 help to drive the specification of Olig2 lineage from an early precursor? Does the compound increase the survival of Olig2 cells over time? Does Y-27632 enhance self-renewal mechanisms in these neural progenitor cells? Detailed characterization of the mechanism underlying the expansion of Olig2 populations in the presence of Y-27632 is of major developmental relevance and might origin clinical translatable knowledge. One can answer several of these questions by taking advantage of the recently developed Olig2::GFP hESC line (Liu et al., 2011).

4. The exact molecular mechanisms through which Y-27632 induces an increase in the number of human motor neurons remain elusive. The comprehensive characterization of the underlying molecular mechanisms will be of fundamental value to design newer drugs that can more rigorously drive motor neuron generation. Is it possible that Y-27632 is leading other cell types to secrete molecules that later act in motor neurons and their progenitors? It would be relevant to analyse the secretome (Pavlou and Diamandis, 2010; Salgado et al., 2010; Suk, 2010) of these cultures in the presence and absence of Y-27632.

5. Supplementation of cultures with EGF and FGF-2 is a well-established procedure and has been the gold standard approach to expand several populations of neuronal progenitors (Caldwell et al., 2004; Ciccolini and Svendsen, 1998; Conti et al., 2005; Ostenfeld and Svendsen, 2004; Pastrana et al., 2011; Pollard et al., 2009). However, it remains unclear whether EGF + FGF-2 are able to robustly expand human motor neuron progenitors; and if Y-27632 is a more reliable strategy than EGF + FGF-2 at expanding those motor neuron progenitor cells.

6. Human motor neurons grown in the absence of neurotrophic factors were rescued from death upon supplementation with Y-27632 in a dose-dependent manner. What are the mechanisms behind the survival effect induced by Y-27632? Is it the same mechanism already proposed for the survival promoting effects of Y-27632 in dissociated hPSCs? (Chen et al., 2010; Ohgushi et al., 2010; Ohgushi and Sasai, 2011) Detailed characterization of these mechanisms with metabolomic and genomic profiling of motor neurons grown with and

without Y-27632 will be of fundamental value to design newer drugs that can halt motor neuron death.

7. While all eight ROCK inhibitor molecules tested elicited axonal outgrowth, only Y-27632 was capable of inducing motor neuron increases in mixed cultures and promote survival of purified and neurotrophically deprived human motor neurons. This suggests that the motor neuron increasing effects of Y-27632 are ROCK-independent. Can we test other specific inhibitors of the kinases that Y-27632 is likely to be inhibiting at the concentration used and evaluate whether the effects observed are induced through other signalling pathways?

8. In the current work 8 different commercially available ROCK inhibitor molecules were tested under identical conditions and using the same assays, which is probably the most complete study performed so far using this class of compounds. It would be interesting to additionally test other molecules in the family which have recently become publicly available, namely SAR407899, GSK269962, SB772077B dihydrochloride, RKI 1447 dihydrochloride, AS 1892802 and SLX-2119.

9. The effects induced by Y-27632 in the hPSC-derived motor neuron cultures can be of potential relevant application in other forms of neuronal and non-neuronal cultures specified from hPSCs. It would be pertinent to evaluate whether Y-27632 can lead to similar results in hPSC-derived cultures of dopaminergic neurons, cardiomyocytes or pancreatic β-cells, for example.

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