5-2012

INDUCED-PLURIPOTENT STEM-DERIVED NEURONAL PROGENITOR CELLS AS A NOVEL TREATMENT FOR NEURODEGENERATIVE DISEASES

Dennisse A. Gonzalez-Romero

INDUCED-PLURIPOTENT STEM-DERIVED NEURONAL PROGENITOR CELLS AS A NOVEL TREATMENT FOR NEURODEGENERATIVE DISEASES

by

Dennisse Andrea Gonzalez-Romero

APPROVED:

_____
Claudio Soto, PhD. Supervisory Professor

_____
Brian Davis, PhD

_____
Neal Waxham, PhD

_____
Jaroslaw Aronowsky, PhD

_____
Jeffrey Frost, PhD

APPROVED:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

INDUCED-PLURIPOTENT STEM-DERIVED NEURONAL PROGENITOR CELLS

AS A NOVEL TREATMENT FOR NEURODEGENERATIVE DISEASES

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Dennisse Andrea Gonzalez-Romero

Houston, Texas

May, 2012

Acknowledgements

To my parents for their constant efforts to give me all the support to pursue a scientific carrier. My father taught me about self-discipline and passion for work. Because of him when I was little I had more books about mathematics than fairy tales, at that time I regretted it, but analyzing the situation retrospectively I realize that those books primed my life. On the other side, my mom taught me about hard work and love and compassion for others. She was the most enthusiastic about my career always pushing me to go for more, to achieve things that she never even thought about.

To my mentor, Dr. Soto. Thanks Claudio for all the support and for teach me to think out of the box. For teach me about science, politic and strategy, for all the never-written knowledge.

To my lab mates, my friends. Thanks for the intellectual input, for the interesting conversations at lunch time, for putting a smile on my face when everything looked black. Thanks to Morales, Diego, Andrea, Diaz, Abhisek, Marcelo, Mike and many others who had shared their lives with me at the PMD-lab. My special acknowledgements to Sylvia a Gloria for their unconditional support.

To Vincent, my *pololito*. Thanks Babe for rebuking me when I needed to be pushed to keep going. Thanks Babe for all your support, help and love.

A mis padres por su constate esfuerzo por darme todo el apoyo para seguir una carrera en ciencia. Mi padre me enseno sobre autodisciplina and pasion por el trabajo. Gracias a el cuando era nina tuve mas libros de matematicas que de cuentos de hadas, en ese tiempo renege de ellos pero ahora que analizo la situacion en retrospective me doy cuenta de como esos libros influenciaron mi vida. Por el otro lado mi mami me senseno sobre el trabajo duro y amor y compassion por los otros. Ella fue la mas entusiasta sobre mi carrera siempre empujandome a ir por mas, a alcanzar cosas que ella ni siquiera penso que existian.

A mi mentor, Dr. Soto. Gracias Claudio por todo el apoyo and por ensenarme a pensar distinto, por ensenarme sobre ciencia, politica y estrategias, por todo el conocimiento que no esta escrito.

A mis companeros de laboratorio, mis amigos. Gracias chicos por el input intelectual, por todas las conversaciones interesantes a la hora de almuerzo, por poner una sonrisa en mi cara cuando todo se veia negro para mi. Gracias a Morales, Diego, Andrea, Diaz, Abhisek, Marcelo, Mike y muchos otros que han compartido conmigo en el PMD-Lab. Agradecimientos especiales a Sylvia y Gloria por su apoyo incondicional.

A Vincent, mi pololito. Gracias Babe por reganarme cuando necesitaba un empujoncito para seguir adelante. Gracias mi amor por todo tu apoyo, ayuda y amor.

Abstract

INDUCED-PLURIPOTENT STEM-DERIVED NEURONAL PROGENITOR CELLS AS A NOVEL TREATMENT FOR NEURODEGENERATIVE DISEASES

Dennisse Andrea Gonzalez-Romero

Supervisory Professor: Claudio Soto, Ph.D.

Cellular therapies, as neuronal progenitor (NP) cells grafting, are promising therapies for patients affected with neurodegenerative diseases like Creutzfeldt-Jakob Disease (CJD). At this time there is no effective treatment or cure for CJD. The disease is inevitably fatal and affected people usually die within months of the appearance of the first clinical symptoms. Compelling evidence indicate that the hallmark event in the disease is the conversion of the normal prion protein (termed PrP^{C}) into the disease-associated, misfolded form (called PrP^{Sc}). Thus, a reasonable therapeutic target would be to prevent PrP misfolding and prion replication. This strategy has been applied with poor results since at the time of clinical intervention substantial brain damage has been done. It seems that a more effective treatment aimed at patients with established symptoms of CJD would need to stop further brain degeneration or even recover some of the previously lost brain tissue. The most promising possibility to recover brain tissue is the use of NPs that have the potential to replenish the nerve cells lost during the early stages of the disease.

Advanced cellular therapies, beside their potential for cell replacement, might be used as biomaterials for drug delivery in order to stimulate cell survival or the resolution the disease. Also, implanted cells can be genetically manipulated to correct abnormalities causing disease or to make them more resistant to the toxic microenvironments present in damaged tissue.

In recent years cell engineering has been within the scope of the scientific and general community after the development of technologies able to “de-differentiate” somatic cells into induced-pluripotent stem (IPS) cells. This new tool permits the use of easy-to-reach cells like skin or blood cells as a primary material to obtain

embryonic stem-like cells for cellular therapies, evading all ethical issues regarding the use of human embryos as a source of embryonic stem cells.

The complete work proposes to implant IPS-derived NP cells into the brain of prion-infected animals to evaluate their therapeutic potential. Since it is well known that the expression of prion protein in the cell membrane is necessary for PrP^{Sc} mediated toxicity, we also want to determine if NPs lacking the prion protein have better survival rates once implanted into sick animals.

The main objective of this work is to develop implantable neural precursor from IPS coming from animals lacking the prion protein.

Specific aim 1: To develop and characterize cellular cultures of IPS cells from prp-/- mice. Fibroblasts from prp-/- animals will be reprogrammed using the four Yamanaka factors. IPS colonies will be selected and characterized by immunohistochemistry for markers of pluripotency. Their developmental capabilities will be evaluated by teratoma and embryoid body formation assays.

Specific aim 2: To differentiate IPS cells to a neuronal lineage. IPS cells will be differentiated to a NP stage by the use of defined media culture conditions. NP cells will be characterized by their immunohistochemical profile as well as by their ability to differentiate into neuronal cells.

Specific aim 3: Cellular labeling of neuronal progenitors cells for in vitro traceability. In order to track the cells once implanted in the host brain, they will be tagged with different methods such as lipophilic fluorescent tracers and transduction with GFP protein expression.

Table of Contents

List of Illustrations

List of Tables

I. Introduction

Neurodegenerative diseases (NDs) are a group of brain disorders in which there is significant neuronal cell loss. Some of the most emblematic examples include Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), Amyotrophic Lateral Sclerosis (ALS) and Creutzfeldt-Jakob's disease (CJD). All those diseases are mainly manifested in elderly, with the risk increasing exponentially with age. Since life expectancy has increased substantially in the last century, an epidemic of neurodegenerative diseases is expected in the coming years. It is estimated that the number of cases of AD alone will rise from 5.4 million in 2011 to 15 millions in 2050 [1, 2] if no effective therapies become available.

Currently there are no effective treatments for any ND. To date available therapies only include palliative strategies. This scenario is aggravated by the lack of early diagnostic tools. For this reason we believe it is necessary to develop new therapies aimed to recover brain tissue and functionality even after substantial brain damage is done, which represent the situation when patients are clinically diagnosed.

Stem cells are at the center of intensive investigation as potential cell replacement treatment for a wide number of diseases. They have the potential to be "transformed" into any cell type of the body, being a good biomaterial for the construction of tissue or even whole organs outside the individual for later transplantation. Pioneering work was done using human embryonic stem cells taken from discarded embryos from in vitro fertilization clinics; however the ethical and legal issues associated with the use of human embryos, in addition to the variable and sometimes poor quality of the cells, made the use of human embryonic cells difficult to evaluate their potential in clinical trials. Recently a novel approach is being explored by many groups worldwide after the outreaching discoveries made by Yamanaka and Takahashi in 2006. They reprogrammed somatic cells to an embryonic equivalent stage, which they called induced-pluripotent stem (IPS) cells [3]. Those cells were shown to have self-renewing and developmental pluripotency capabilities. This new technology offers opportunities for autologous cell therapies,

patient-specific drug screening [4] and the development of new in-vitro models of diseases, besides overcoming the ethical aspects of using embryonic stem cells.

The next sections will provide an introduction to neurodegenerative diseases, stem cells and how iPS cells are ideal sources of donor cells for replacement therapies as well as useful tools for *in situ* release of trophic factors or drugs.

I.1.1. Molecular mechanism of neurodegenerative diseases

A common characteristic of NDs is the appearance of protein aggregates (also called amyloids) in the brain parenchyma or inside neuronal cells. The aggregates are constituted of abnormally folded proteins, which vary according to the disease[5] . For example, beta-amyloid peptide is the main component of aggregates (called plaques) in AD; alpha-synuclein in PD; SOD-1, FUS and/or TDP-43 in ALS; huntintin in HD or prion protein in CJD. Regardless of the protein involved the common pathological event involve the re-folding of the normal host protein into a stable misfolded variant which can multimerize with units of the same protein and induce their abnormal reassembly (see box 1). The most current theories of neurodegeneration propose that small oligomers in their aberrant conformation are the most toxic particles for cells by many concurrent mechanisms. For example, studies done in AD suggest that beta-amyloid induce the dysfunction of synaptic activity at the pre- and postsynaptic levels (reviewed in [6]), calcium deregulation due to the formation of pores in the plasmatic membranes and activation of apoptotic pathways. Also, cells experimenting distress due to fibrillar forms of beta-amyloid peptide or prions activate the unfolded protein response (UPR) inducing the down down regulation of the general expression of proteins but overexpression of molecular chaperons leading eventually to apoptotic death (rev in [7]).

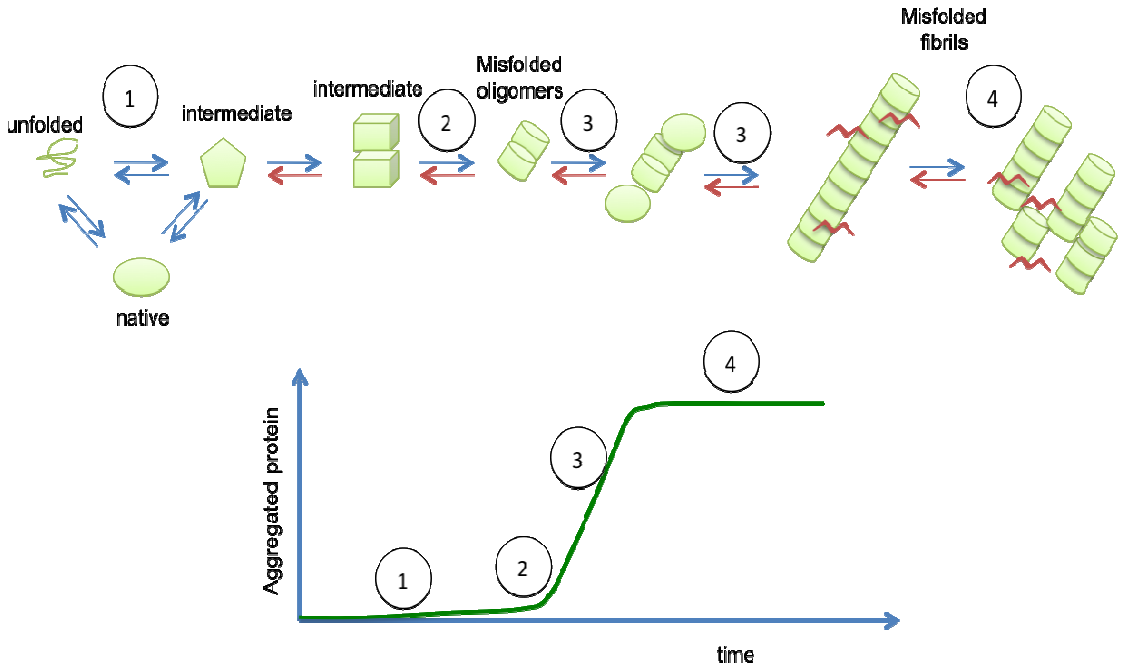
I.1.2. Prion Disorders as a model of Neurodegenerative diseases

Prion diseases, also called Transmissible Spongiform Encephalopathies (TSEs), are a group of fatal progressive neurological disorders affecting humans and other mammals like deer, sheep, cattle, minks, and others. Human TSEs include CJD (familial, variant and iatrogenic forms), kuru, fatal familial insomnia and Gerstmann-Straussler-Scheinker syndrome. Clinical symptoms are variable depending on the disease, but include loss of motor coordination, dementia, ataxia, vision impairment, and sleep disturbances. The disease is inevitably fatal and affected people usually die within months of the appearance of the first clinical symptoms. At the pathological level, the brain of affected individuals shows vacuolation, extensive astrogliosis and the accumulation of prion protein aggregates.

Prion protein is a glycoprotein of around 210 amino acids encoded by a single gene (prnp). It harbors a glycophosphatidylinositol (GPI) motif that binds to the external side of the plasma membrane. There are still no clear physiological functions attributed to the prion protein, however data suggest that might be participating in cell adhesion, copper uptake, protection against oxidative stress, modulation of neuronal excitability, regeneration of adult muscle tissue, and regulation of apoptosis [8-12]. The current model of prion pathogenesis indicates that the normal host prion protein (called PrP^{C}) converts into a disease-associated conformation (called PrP^{Sc}). In its abnormal conformation PrP^{Sc} is able to recruit PrP^{C} and act as a corrupt template inducing the conversion toward PrP^{Sc} conformation. The dynamic of misfolding follows a seeding-nucleation model of polymerization, including a slow phase where the primary PrP^{Sc} seeds are formed and a fast phase when the polymerizing events take place to finally form fibrillar structures of different sizes (see box 1). In vivo, the initial step of PrP^{Sc} formation might be facilitated by mutations that make PrP^{C} prone to misfold and form the primary seeds; these events could explain the cases of familial prion diseases. Also, the formation of PrP^{Sc} in wild type animals can be induced by the administration of PrP^{Sc} from infected tissues.

A very important contribution to understanding the mechanism of prion diseases was made with the development of prion protein knockout mice. Mice lacking the prion protein were shown to be viable without any dramatic phenotypic alteration while they were resistant to prion infection. This result strongly suggested that PrP^{C} expression is absolutely necessary to develop the disease [13]. In accordance with this result, the reintroduction of the prion protein in the prp-/- mice restores the infectivity of PrP^{Sc} [14]. Further studies in mice showed that the brain tissue of prp-/- mice is resistant to PrP^{Sc}-mediated toxicity [15]. Furthers studies also indicated that the prion protein must be attached to the cell membrane in order for the cells to be susceptible to prion toxicity. Indeed, Chesebro et al. showed that transgenic animals expressing the prion protein without its GPI anchor do not develop the clinical disease, but are still able to replicate PrP^{Sc} [16]. Similar results were obtained in vitro where cells expressing the anchorless prion protein were shown to be resistant to prion infection [17]. Taken together this data supports the hypothesis that neuronal stem cells lacking the prion protein might be more resistant to prion toxicity in vivo when implanted into a diseased animal.

Many lines of evidence suggest that other NDs follow a prion-like mechanism of protein misfolding [18]. For example there is substantial evidence suggesting that beta-amyloid peptide misfolds following the same kinetics as prions and that it could be also transmissible between individuals [19-21] as well as between neurons. Similar findings have been published suggesting that ALS [22] and Parkinson's might follow the same mechanism. An interesting example of prion-like spreading of misfolded proteins was observed in postmortem analysis of Parkinson brains that were grafted with healthy embryonic neurons. A subset of the implanted cells harbored Lewy bodies (aggregates mainly composed of alpha-synuclein), suggesting that the misfolding events took place after the cells were in contact with the misfolded alpha-synuclein seeds present in the Parkinsonian brain [23-25]. Further experiments showed that alpha-synuclein is internalized by neuronal stem cells when they are implanted in an animal model of Parkinson's[26], suggesting a cell to cell transmission of the oligomeric, misfolded particles.



Box1**. Kinetics of prion-like protein misfolding.** Proteins showing high tendency to self aggregation in a prion-like fashion contain hydrophobic motifs that tend to self-interact forming unstable intermediate isoforms (1). When a critical number of monomers interact forming a stable seed (2), the misfolding process accelerates exponentially (3) until the majority of the native protein has been converted to the abnormal conformation (4).

I.1.3 Therapeutic alternatives for protein misfolding disorders

The most promising treatments against neurodegenerative disorders target the protein misfolding process. Along these lines pioneering work headed by Dr. Soto showed that beta-sheet breaker (BSB) peptides, (short peptides with high homology to amyloidogenic regions of fibrillogenic proteins but harboring proline as beta-sheet destabilizing residues), are able to avoid the formation of amyloid fibrils and re-dissolve existing ones. BSBs designed for beta-amyloid peptide and prions

were shown to be effective in vitro and in vivo [27-30]. However, due to their peptidic nature, they were extremely labile when administered systemically. We believe that BSB, secreted from grafted cells, can be a useful tool to fight the formation of amyloid deposits already existing in the brain, as well as to help stop the misfolding of new proteins.

I.2.1. Stem cells

Stem cell is the general term to describe self-renewing cells able to differentiate to many specific cellular types. Embryonic stem cells (ESC) are present in the inner mass of the blastocyst and can give rise to any embryonic tissue, therefore called “pluripotent cells”. Adult tissue harbors “adult stem cells” which are progenitor cells able to give raise to a restricted spectrum of cell types, hence called “multipotent” progenitors cells.

For more than five decades researchers have tried to generate pluripotent cells from somatic cells. The first “cloning” technology was described by King and Brigs in the 1950’s; they and others showed that by transferring the nuclei of a late-stage embryonic cell into an enucleated oocyte it is possible to “re-set” the genetic information indicating that the changes in the DNA through development are of epigenetic origin [31]. Dolly the sheep was the first successful case of somatic cell nuclear transfer of mammalian cells completing embryonic development; this finding indicated that the genome of fully differentiated somatic cells retain their “totipotential” capabilities [32] even after having undergone full differentiation to a specific cell line. Although Dolly was fairly healthy, other cases of mammalian cloning exposed abnormal phenotypes and gene expression pattering [33, 34] implying that epigenetic reprogramming is not thorough after cloning. A revolutionary contribution was made by Takahashi and Yamanaka in 2006 [3] when they identified four transcription factors (Oct-3/4, Sox-2, Klf-4, C-Myc) that when transiently overexpressed in murine fibroblasts are able to drift the phenotype towards an embryonic character. Those cells were shown to be pluripotent in their

ability to differentiate and self-renewing, hence called by the authors pluripotent-induced stem (IPS) cells. A year later the team led by Jaenisch [35] showed that IPS cells are able to complete embryonic development leading to healthy, normal animals, indicating that IPS cells suffer a complete nuclear epigenetic reprogramming ending with similar developmental capabilities to embryonic stem cells.

The use of IPS cells as a source of autologous cells for therapies offers the possibility to bypass the complications due to immune rejection and the use of immunosuppressants. Also, they overcome the ethical issues associated with the use of stem cells of embryonic origin.

Rodent and human IPS cells have been successfully differentiated into functional adult cells. Chambers and others were able to differentiate human IPS cells toward neural progenitors with high efficiency by inhibiting the SMAD signaling pathway [36]. Also, motor neurons [37, 38], dopaminergic neurons [39], retinal cell [40], hepatocytes [41], blood cells[42], and adipocytes [43] have been successfully obtained after in vitro differentiation of human IPS cells. With a similar approach used for IPS cells generation, it is also possible to transdifferentiate fibroblasts directly to neuronal progenitors [44] or neurons [45].

In the last four years substantial advances have been made to develop better methods to produce high-quality IPS cells for their potential use in clinical settings. The initial reprogramming experiments were done transducing the somatic cells with genome-integrating viruses; however the disruption of the integrity of the host DNA is a potential risk for the later development of tumorogenic cells. Different delivery systems have been designed in order to avoid insertional mutagenesis, which is the main risk for any kind of DNA-based vector. Later technical advances include the use of recombinant proteins and mRNA for cell reprogramming. Table 1 summarizes the reprogramming methods available to date focusing on their advantage and disadvantages.

The low rate of efficiency of all reprogramming systems is one of the main obstacles in the generation of human IPS cells. However, this can be improved by

the use of small molecules like the MEK inhibitor PD0325901, the ALK5 inhibitor SB431542 [46] and valproic acid, among other molecules.

Method of delivery	Advantages	Disadvantages	Refs
Retroviral vectors	High efficiency (0.01-0.5%)	Insertational mutagenesis Incomplete silencing	[3]
Adenoviral vectors	Low frequency for integration	Low efficiency (0.001%)	[47]
Lentiviral vectors + Cre	Higher efficiency (0.1-1%) Infects non-dividing cells	Insertational mutagenesis loxP sites remain integrated	*(7)*
PiggyBac transposons	Precise deletion possible	Low efficiency (0.001%)	[48]
DNA plasmids	Low frequency for integration	Extreme low efficiency	[49] [50]
Mini-circle DNA	Low frequency for integration	Low efficiency (0.005%)	[51]
Proteins (arginine peptide tagged)	No genetic modification Simple protocol	Low efficiency (0.001%) Require many transfection cycles	[52] [53]
mRNA	No genetic modification High efficiency (1%)	Require many transfection cycles	[54]

Table1. **Delivery methods of reprogramming factors.** Values of efficiency from [55].

I.2.2. Stem cells as a therapeutic tool

Due to the developmental potency of stem cells, they offer a promise for cell grafting therapies. One of the most explored cell therapies in humans to date is the

transplant of autologous mesenchymal bone marrow stem cells (BMCs) into the failing heart. BMCs have been shown to reduce considerably the mortality of patients affected with acute infarction and chronic ischemic heart failure [56]. Initially the mechanism of action was attributed to the regeneration of myocardial tissue by BMCs, however later evidence indicates that the effects are mainly mediated by the release of paracrine factors that improve the function of already existing cardiomyocites or promoting the development of cardiac stem cells (reviewed in [57]).

BMCs have also been administered to stroke patients. Several clinical trials are currently in place to determine the efficacy of BMCs when administered intravenously. Many questions have arisen regarding the mechanism of action of peripherally administered stem cells; In vitro data suggests that BMCs might differentiate directly into neurons in the brain; however later data has shown that the population of implanted cells that reach the target tissue is too low to produce any type of direct effect. Other lines of evidence suggests that grafted cell release beneficial factors like anti-inflammatory cytokines and growth factors able to act as paracrine signals positively influencing the clinical outcome.

Neural stem cells, which can be derived in vitro from ES and IPS cells, have also been implanted in animal models of neurodegeneration. Yamasaki et al. reported memory improvement of animals suffering from neuronal loss primarily in the hippocampal area. They showed that neural stem cells are able to survive, migrate and differentiate in the host brain [58]. A later report from the same group indicated that the cognitive rescue due to neural stem cell transplantation is mediated by brain-derived neurotrophic factor (BDNF) [59] which is released by the implanted cells. In parallel, neural stem cells have also been implanted in animals infected with prion diseases. The results indicated that animals receiving neural stem cells lived longer compared to prion infected animals receiving only the vehicle [60].

As discussed before stem cell therapy opens new avenues for the development of new therapeutic alternatives; however until now only a few studies using IPS cells in models of neurodegenerative diseases are available.

I.3.1 Hypothesis

It is possible to generate IPS cells from animals lacking the prion protein and induce their differentiation into neural precursors.

I.3.2. Objectives

For my dissertation work I have identified three objectives:

1. Development and characterization of IPS cells from prp-/- somatic cells

2. In vitro differentiation of IPS cells into neural precursors and fully differentiated neuronal cells.

3. Cellular labeling of neural precursor cells for in vivo traceability.

II. Materials and Methods

Animals

Wild type 129S2/SvHsd and *prp*-/- (from here also called KO) transgenic mice aged 12 months were used for primary culture of fibroblasts. Prp-/- mice were developed by C. Weissmann [13] in a 129S2 background.. The spring was genotyped by PCR (5'AGCTGTACAAGAAAAAGCGGC; 3' CTTGCTCACCATGCAGAGGCC). Stereotaxic injection of cells was conducted under deep anesthesia by inhalation of 5% isofluorane for induction and 2% during the surgical procedure. Animals were sacrificed using CO_2 inhalation according to the protocol approved by the Animal Welfare Committee of UTHSC-H.

Primary culture of tail tip fibroblasts

To obtain fibroblast culture from tail tip we followed the protocol described in [61] with few modifications. Briefly, tail tips from wild type and transgenic mice were soaked in alcohol and then piled from external skin and chopped in 0.5 cm length pieces using sterile material. Then, 3 to 5 tail pieces were culture for one day in a 3.5 cm gelatin-coated culture dish containing a thin layer of basic media (DMEM, 1x antibiotic-antimycotic solution containing Amphotericin B, Streptomycin, and Penicillin (Invitrogen), 2 mM L-glutamine and 10% fetal bovine serum). On the second day more media was carefully added trying to avoid lifting the tail pieces until a total of 2 ml of media per well. From there, the media was changed every other day until the emerging fibroblasts reached confluence.

For feeder cells, embryonic mouse fibroblasts (MEFs) were harvested from mice fetuses following the procedure of Dr. Ana Crane (protocol not published). Briefly, fetuses were removed from the uterine horns of 13.5 days pregnant female mouse. Fetuses were washed three times with PBS and then the limbs, head and visceral tissue was removed and disposed. The remaining tissue was thoroughly

minced and then incubated in 0.05% trypsin/EDTA for 30 min at 37^{o}C. After enzymatic digestion with trypsin most of the cells were found in fine suspension although some chunks of tissue was also observed. Next, cells were resuspended in basic media, plated in tissue culture flasks and left in a cell tissue incubator. Cells were passed three times with trypsin when they reached at least 90% confluence. To mitotically inactivate the cells, they were trypsinazed and pooled in a 50 ml conical tube to then be irradiated at 8000 RAD (this step was done by Dr. Crane). Finally cells were counted and resuspended in cryopreservation media (DMEM plus 10%DMSO and 10% FBS) for long term storage in the vapor phase of liquid nitrogen.

Culture of Stem cells

ES and IPS cells were culture over a feeder layer of mitotically inactive MEFs (iMEFs) cells following the protocol provided by Stemgent for mouse IPS cells [62]. Briefly, plates were incubated with 0.2% gelatin overnight at room temperature in sterile conditions. Next day iMEFs cells were thawed and seeded at 1x10^{5} cells/cm^{2} in basic media, and then they were incubated overnight at 37^{o}C with 5%CO_2 . The following day basic media was removed, cells were washed twice with PBS and finally they were incubated in ES media (KO-DMEM media supplemented with 15% mESC-qualified fetal bovine serum (Atlanta biological), 2x10^{4} units/ml leukemia inhibitory factor (LIF), 2 mM L-glutamine, 1x non-essential amino acids, and 0.1 mM **β-**mercaptoethanol) for at least 2 hours prior plating ES or IPS cells. Everyday ES media was removed from the plate and fresh pre-warmed media was carefully added. ES and IPS cells were monitored daily to look for signs of spontaneous differentiation or excessive density of colonies in the plate. Signs of differentiation include the presence of merged colonies containing cells of abnormal morphology. If differentiated cells were spotted, they were mechanically removed by aspiration with a Pasteur pipette or the complete dish was discarded.

ES and IPS cells were split before colonies appeared to merge. For that the media was removed and the cells were washed twice with PBS (without Calcium/Magnesium). Then, 1 ml of prewarmed 0.05% trypsin/EDTA solution was

added to 1 well of 6-wells plate and the plate was incubated for 5 min at 37^{o}C. We added 1 ml of ES media to neutralize the trypsin and then the cells were pipette up and down to create a single cell suspension. Next, cells were transfer to a 15 ml conical tube and other 5 ml of media were added to the tube. Finally cells was centrifuged at 150xg for 5 min, then resuspended in fresh ES media and 1/6 of total cells were transfer to a new well containing a feeder layer of iMEFs.

For cryopreservation, ES and IPS cells were trypsinazed as described for passaging and then resuspended in cryopreservation media (KO-DMEM supplemented with 10% FBS and 10% DMSO). Then 1 ml of suspension was transferred to each cryovial. Finally cryovials were stored in a freezing container for 24 hours at -80^{o}C and the next day vials were transferred to a liquid nitrogen tank for long-term storage.

Control mouse IPS cells were purchase from Stemgent (cat 08-0007), reprogrammed from fibroblast with a retrovirus encoding the Oct-4, sox-2, Klf-4 and C-Myc transcription factors produced in Dr. Jaenisch laboratory [63].

Production of iPS cells

Reprogramming of adult fibroblast was carried out by transduction with the four Yamanaka factors following the same basic protocol [3, 61]. Briefly, fibroblasts were seeded over gelatin-coated plates at 1x10^{5} cells in one well of 6-wells plate in basic media and incubated overnight in a tissue culture incubator. Next day, one well of cells was incubated in transduction media (basic media plus the Oct-4, Sox-2, Klf-4 and C-Myc ecotropic retrovirus. Retroviruses from Stemgent, cat 00-0042) and another well of fibroblasts was incubated in basic media plus GFP ecotropic virus to assay efficiency of transduction. Transduction media was supplemented with 4 µg/ml polybrene and plates were centrifuged at 1000xg for 30 min to maximize the efficiency of transduction. After 24 hours of incubation, transduction media was removed and discarded and fresh basic media was added to the cells. Virus-contaminated liquid were disposed after inactivation of biohazard material by incubation for at least 20 minutes in 20% bleach. Solid-contaminated waste was

collected in closed biohazard bags for their immediate disposal in order to avoid the formation of dry volatile virus-containing particles. For the same reason, all the equipment used was cleaned with 10% bleach. Next day cells were passaged and plated at $1x10^4$ cells per cm^2 in a gelatin-coated plate in basic media. After 24 hours the media was changed to ES media and then fresh media was added every other day. Cells were monitored daily for the appearance of colony with similar morphology to ES cells.

Characterization of IPS cells

Pluripotent cells were characterized by staining with Alkaline Phosphatase detection kit (Millipore) following the recommendations of the manufacturer. Briefly, cells were incubated for 15 minutes in a solution containing two parts of Fast Red Violet solution (0.8g/L stock) and one part of Napthol AS-BI phosphate solution (4mg/mL) in AMPD buffer (2mol/L), pH 9.5. Then, cells were washed three times with PBS and finally cells were analyzed under a bright field inverted microscope (Motic AE30).

For the embryoid bodies formation assay, IPS cells were cultured for 3 passages over gelatin-coated plates to get rid of the feeder cells. Then the pluripotent cells were grown in the presence of basic media in microwells following the guidelines provided by the manufacturer (Aggrewell 400, Stemcell Technologies). Briefly, plates containing microwells were filled with DMEM/F12 media and centrifuged at 2000xg for 5 minutes in order to remove bubbles. After the DMEM media was removed, a suspension of $1,2 \times 10^5$ cells was added to each well and the plate was centrifuged to 100xg for 3 min to pull own the cells to the bottom of the microwells. After 24 hours of incubation at 37^oC, 5% CO_2 the media was gently replaced for fresh one. After 48 hours past the initial incubation cells were lifted off from the bottom of the well by slowly pipetting up and down. The aggregates of cells were passed through a 35 µm cell strainer in order to retain the aggregates while wash out single cells. Aggregates were washed three times with PBS and then collected in a conical tube by inverting the strainer. Finally the cell aggregates were plated in ultra-low adherence wells in basic media and incubated

for another 2 weeks. Media was changed every other day by removing half of the volume with a Pasteur pipette and adding the same volume with fresh pre warmed media.

For the teratoma formation assay wt and mutant feeder-free IPS cells were harvest by trypsinization. The reaction was stopped by dilution in PBS and cells were counted. Next, cells were centrifugated at 100xg for 5 minutes at room temperature and the concentration was adjusted to $1x10^5$ cells/µl in DMEM supplemented with antibiotic/antimycotic solution. Cells were kept in ice until use. Animals were anesthetized with by inhalation of 5%-2% isofluorane. After the animals were unresponsive to physical pain it was placed in a sterotaxic apparatus with constant supply of 2% isofluorane. Then, the upper are of the head of the animal was shaved and a small incision was made (0.5-0.8 cm). Then, a small orifice was drilled in the cranium at 2 mm posterior of bregma and 2 mm right of the middle line. Next, using a Hamilton syringe 2 µl of cells was injected in the hippocampal area of the right hemisphere. Finally the incision was stitched with silicon coated braided silk (Softsilk) and the animal was placed over a warm pad until recover from anesthesia. Animal were sacrificed after 3 weeks and brain tissue was fixed for histological analysis.

Differentiation of iPS into neural precursor cells

For differentiation to neuronal precursors cells, feeder-free IPS cells were culture over gelatin-coated plates in ES media for at least 3 passages. Then, cells were treated under different protocols:

Protocol 1 [64]: Cells were resuspended in cellular aggregates media containing DMEM, 10%FBS, 2mM L-glutamine, 1x non-essential amino acids, and 0.1 mM beta-mercaptoethanol in a low-attachment plate. Media was changed after 2 days. At the 4^{th} and 6^{th} day the media was changed and supplemented with 5µM retinoic acid (RA. Sigma). At the 8^{th} day the spheres were disrupted by enzymatic digestion with 0.05% trypsin/EDTA and reseeded in a poly-ornithin/laminin coated dish in N2 media (DMEM/F-12 plus 25 ug/ml insulin (sigma), 50ug/ml human apo-transferrin (Sigma), 20 nM progesterone (Sigma), 100 nM putrescine (sigma), 30 nM sodium selenite (Sigma), 50 ug/ml BSA (Sigma), pH 7.0-7.8).

Protocol 2 [65]: Cells were resuspended in DMEM/F12 –Neurobasal (Gibco) (1:1) medium supplemented with (25 µg/ml insulin, 100 µg/ml apo-transferrin, 6 ng/ml progesterone, 16 µg/ml putrescine, 30 n*M* sodium selenite) plus 50 µg/ml bovine serum albumin fraction V, and B27 supplement (invitrogen) and replated in a gelatin-coated plate. Media was changed every day for at least 6 days.

Protocol 3 [66]: Cells were resuspended in Neural precursor (NP) media containing DMEM-F12 media (Invitrogen) and Neurobasal (Gibco) (1:1) medium supplemented with 1x N2 (Invitrogen), 1x B27 (Invitrogen), 15 ng/ml basic fibroblast growth factor (bFGF, stemgent), 50 ng/ml epidermal growth factor (EGF, stemgent), 50ug/ml apotransferrin (Sigma) in a gelatin-coated dish. Media was changed every day for 6 days and then cells were passaged by digestion with 0.05% trypsin/ETDA for 1 min at 37^{o}C. To neutralize the enzymatic reaction, cells were diluted 5x in PBS containing calcium and magnesium, centrifugated for 5 min at 100xg and then resuspended in NP media to finally re-seed in a gelatin-coated dish.

Protocol 4: Cells were resuspended in NP media supplemented with 5uM RA and plated in a gelatin-coated dish. The second day media was replaced with the same formula. At day 3rd and 4th media was changed and replaced with NP media supplemented with 3 uM RA. At day 5th and 6th madia was changed and replaced with NP media supplemented with 1 uM RA. Between every media change cells were washed with PBS to eliminate non-attached cells. At day 7 cells were detached by incubation with 0.05% trypsin/ETDA for 1 min at 37^{o}C. To neutralize the enzymatic reaction, cells were diluted 5x in PBS containing calcium and magnesium, centrifugated for 5 min at 100xg and then resuspended in NP media supplemented with 1uM RA to finally re-seed the cells in a gelatin-coated dish for expansion.

Neural precursor cells were storaged in cryopreservation media containing DMEM/F12 media supplemented with N2, B27 and 10% DMSO. Vials were slowy

frozen at -80^{o}C in freezing container and then stored in the vapor phase of liquid nitrogen.

Differentiation of neural precursors into neurons

For neuronal differentiation NP cells were cultured over L-ornitin/laminin plates in the presence of NP media lacking bFGF and EGF changing the media every two days. For glial differentiation cells were cultured over gelatin-coated plates in the presence of NP media lacking growth factors but supplemented with 1% fetal bovine serum [66].

Labeling of neural precursor cells

Neural precursor cells were seeded at 1x10^{5} cells/cm^{2} in a gelatin-coated plated. Next day cells were transduced by adding EGFP-retrovirus produced in the lab. For that packaging 293T derived cells transfected with pol and gag genes (Platinium-E cells, CellBiolabs) were cultured in basic media in the presence of 10 µg/ml blasticidine and 1 µg/ml puromycine for selection. Cells were passaged by trypsinization when they reached 70% confluence. One day before transfection cells were seeded at 6x10^{4} cells/cm^{2} in absence of antibiotics. Next day we transfected the Platinium-E cells with 2 µg of pMXs-IRES-GFP vector (for a 3.5 cm well) using a lipophilic carrier (X-treme, Roche). After 48 hours the supernatant was collected and passed through a 45 µm pore filter. Then it was centrifuged at 26000 rpm for 2 hours at 4^{o}C in a 70-Ti rotor (Beckman). After centrifugation the supernatant was eliminated and NP media was added to the pellet that contained the retroviral particles. The solid pellet soaked in media was left overnight at 4^{o}C after which the pellet was disaggregated. The virus-containing media was added to the neural precursors at several concentrations and polybrene was added to some wells at 4 µg/ml final concentration. Also, some wells were centrifuged at 1000xg for 30 min after the addition of the viruses. Next day the media was changed for fresh virus-free NP media and from there the culture was treated as normal neural precursor cells.

Immunochemistry

Cells were fixed in the plates with 4% paraformaldehyde and then post-fixed with ice cold 100% methanol for 20 min at -20°C. Then, cells were blocked for 1 hour at room temperature with Tris buffer solution (TBS) supplemented with 0.25% triton X-100, 3% BSA and 3% normal donkey serum. After 2 washes with TBS, cells were incubated overnight at 4°C in the presence of primary antibody diluted in TBS plus 0.12% triton X-100 and 3%BSA. Antibodies used in this study includes: SSEA-1 coupled to PE (Stemgent), Oct-3/4 (Stemcells Technologies), Musashi (Cell Signalling), PAX-6 (DHSB), SOX-2 (R&D), Vimentin (DHSB), and TujI (Chemicon). Next day cells were washed again with TBS and secondary antibody was added at a 1:400 concentration for 1.5 hours at room temperature using the same diluting solution as done for incubation with primary antibody. Next, cells were washed 3 times with PBS, then incubated for 5 min in DAPI solution for nuclear staining and washed again. Finally, a drop of mounting media was added and then covered with a round coverslip before image acquisition. All pictures of fluorescent samples were taken using a BFC360 FX camera coupled to a DMI6000B Leica microscope. Phase contrast pictures were taken using a Moticam 2300 camera counted over a Motic AE30 microscope.

Electrophoresis and Western blotting

Protein electrophoresis was performed in a 12% bis-tris acrylamide gels. Gel were western blotted against a nitrocellulose membrane for 1 hour at 400 mAmp. Then, the membrane was blocked with 5% milk in washing buffer (PBS plus 0.05% tween-20) for 1 hour at room temperature. Then the membrane was incubated overnight at 4°C with antibody 6D11 (Covance) diluted 1:5000 in washing buffer. Next day the membrane was washed three times 10 minutes each with washing buffer to then be incubated in a dilution of sheep anti-mouse IgG coupled to horseradish peroxidase (GE). After other 3 washes, we added an appropriated substrate for horseradish peroxidase (ECL Plus Western Blotting Detection System, GE) and the chemiluminescent signal was captured in a Chemidoc dark chamber (Bio-Rad).

Cell sorting

For sorting of IPS cells they were detached from the plates by digestion with 0.05% trypsin/EDTA, centrifugated at 150xg for 5 min and resuspended in cold sorting solution containing Hank's salt buffer solution (HSBS) without calcium or magnesium supplemented with 10 mM HEPES buffer, 2% heat-inactivated ESC-qualified FBS , 2 mM glutamine, non-essential amino acids, $2x10^4$ units/ml LIF and 0.1 mM β–mercaptoethanol, 1x antibiotic-antimycotic solution containing Amphotericin B, Streptomycin, and Penicillin (Invitrogen). Next, cells were taken to the Cytometry and Cell Sorting Facility at Baylor College of Medicine for cell sorting. IPS cells were sorted using a FACSAria II sorter (BD Biosciences) equipped with a yellow-green (561 nm) laser for PE excitation. Neural precursors were sorted using a FACSAria equipped with a blue (488 nm) laser. After sorting cells were collected in the appropriated cold media (ES or NP media) and then culture under standard conditions.

III. Results

III.1. Prp-/- mice do not express the prion protein

The animals were genotyped to confirm the lack of the ORF for *prnp* gene in their genome. We performed a conventional PCR using the primers listed in material and methods section. As expected we observed one band in wild type mice but not in KO mice (figure 1C), confirming that the gene coding for prion protein is not in the genome of the transgenic mice. Prion protein is mostly expressed in brain cells so we wanted to check the expression of PrP in the brain of KO prp mice in comparison to wt mice. We homogenized the brain of wild type and Prnp Ko animals in PBS plus a cocktail of proteases inhibitors at 10% (w/v). Then the samples were submitted to electrophoresis in a 12% denaturant Bis-Tris acrylamyde gel followed by western blotting. We observed the three typical bands for PrP using the 6D11 antibody in wild type mice and the lack of it in KO animals

(figure 1A). In parallel we ran another electrophoresis and the gel was stained with coomasie blue to confirm that we loaded the same amount of proteins (figure 1B).

III.2. Generation of primary culture of fibroblasts from prp-/- and wild type mice

As described in the material and methods section, fibroblasts were cultured from the tails from 1 year old wild type and prp-/- mice. Only tail pieces attached to the bottom of the well were able to give rise to new cultured cells; the rate of attachment was similar in both set of samples. The most critical factor to obtain a high rate of attachment of growing cells was the experimental manipulation when adding media more than the cell type. Figure 2 illustrates the development of fibroblasts from KO tails

III.3. Cellular reprogramming of terminally differentiated fibroblasts into induced pluripotent stem cells.

To reprogram the fibroblast from prp-/- mice we used the protocol described by Yamanaka consisting on the overexpression of four transcription factors [61]. For that, we transduced the fibroblast at passage 3 with 4 ecotropic retroviruses coding for Oct-4, Soc-2, Klf-4 and C-Myc. As a control of transduction we infected another set of cells with the same vector encoding only for GFP (Figure 3A). We observed a transduction efficiency of 25% as evaluated by microscopy after 48 hours post transduction. A second measurement of the quantity of transduced cells was made by staining a subpopulation of transduced fibroblasts with antibodies against the ectopic genes Sox-2 and Oct-4 (figure 3B-E). We observed that 22% of cells were positive for Sox-2 and 27% were positive for Oct-4. Taking together our results indicate that around 25% of cells express at least one of the ectopic reprogramming factors.

Two days after transduction, cells were passaged and reseeded in gelatin-coated wells. When they reached 70% confluence media was changed to ES media which contain LIF, a inhibitory cytokine of cell differentiation of mouse pluripotent cells [67]. Soon after (two days) media switch we observed the emergence of

clusters of cells with abnormally high cytoplasm/nucleus ratio (fig 4A); these types of formations are described by other groups as primordial reprogrammed cells, which include semi and fully undifferentiated cells. Some of these clusters were transients, dying after 2-3 days. After 10 days of adding the reprogramming media we were able to observe colonies morphologically reminiscent of mES cells. The colonies have bright and smooth edges, with no observable separation between cells (Figure 4B). We also observed the formation of amorphous non-reprogrammed colonies and rapidly growing KO fibroblasts (Figure 4B). We did not observe cells of abnormal morphology or abnormal growth rate in the cells treated with the GFP-retroviral vector (fig 4C).

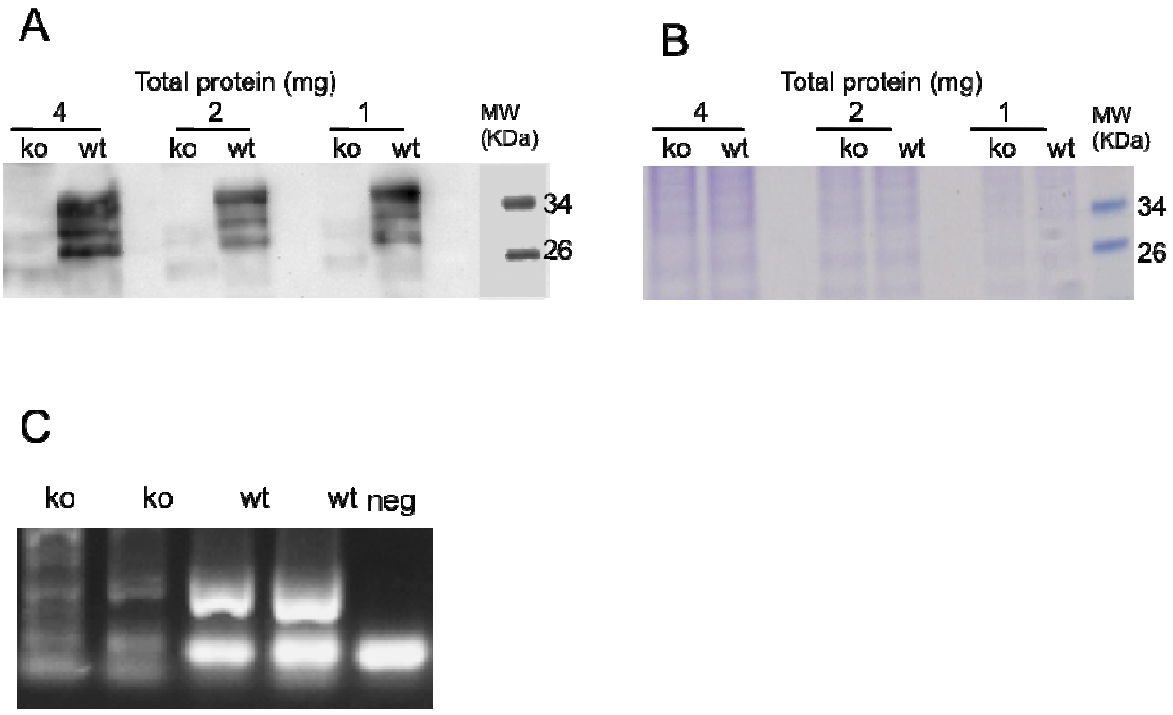


Figure 1. **PrP is expressed in wild type mice but not in prp -/- mice**. (A) Crude homogenate of KO prp and wt mice brain were western blotted against the prion protein (6D11 antibody 1:5000 dilution). We observed the three glycoforms of the prion protein in wild type but not in KO brains. (B) Same samples were stained with coomasie to confirm equal amount of total protein. (C) DNA extracted from tails of KO and wild type mice were submitted to PCR to amplify a segment of the prp gene. We observed an amplification of DNA in wild type mice but not in KO mice. Each line represents different animals. Last line corresponds to negative control (PCR reaction mix without DNA).

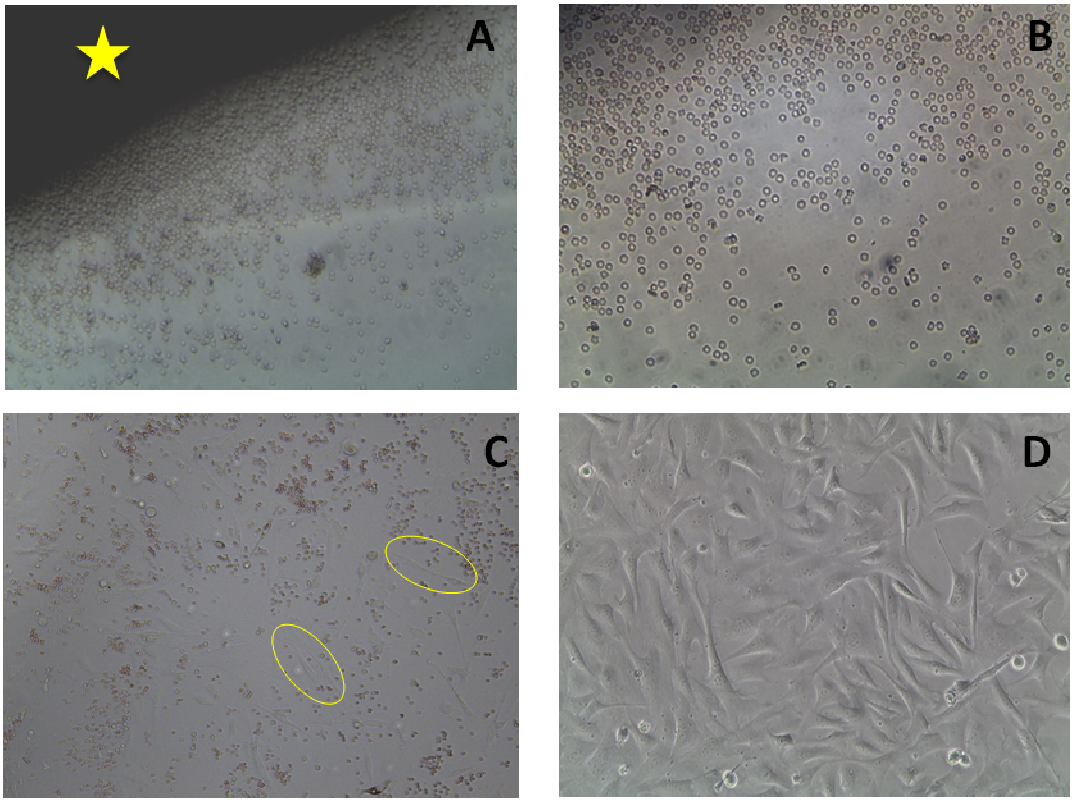


Figure 2. **Primary culture of prp-/- fibroblasts**. Phase contrast photos of the development of primary fibroblasts from adult prp-/- tail tips. Fragments of tail were seeded in gelatin-coated wells. After two days we observed small rounded immature cells growing off the piece of tail (marked with a yellow start star in Panel A, 100x magnification). Panel B shows a detail of the nascent cells from A (200x magnification). After 5-6 days the rounded cells started to elongate like the cells shown inside the yellow ovals, panel C. After 8-9 days cells reached confluence (panel D, 100x magnification) and were passaged using 0.05% trypsin/EDTA. We did not observed any difference between KO and wild type cultures.

In order to isolate mES-like colonies we manually detached the cells from the plate using a rounded tip glass Pasteur pipette. Then they were collected and manually disaggregated to obtain a single cell suspension. Finally cells were reseeded over a layer of irradiated MEFs preconditioned with reprogramming media. After two manual passages of mES-like colonies on feeder cells we

performed the alkaline phosphatase (AP) assay for pluripotency. AP is an enzyme expressed in embryonic stem cells and other cell types, being an unspecific but well accepted marker for screening of pluripotency [68]. To carry out the test cells were incubated for 15 min in the dark in the presence of a diffusible substrate for alkaline phosphatase turning the cells a violet color after the reaction takes place. We observed that only 34 ± 9.2 % (SE) (n=7 colonies, 376 cells) of the cells belonging to a same colony were positive for AP (fig 5A-C). We performed a second test for pluripotency, in this case we stained the live cells with an antibody for the surface protein SSEA-1[69]. In agreement with the previous test we observed that only a subset of cells was positive for SSEA-1 (Fig5 D-F). We estimated that the percentage of positive cells is 34.2 ± 12.6% (SE) (n=7 colonies, 363 cells). The same population of cells was submitted to flow cytometry to detect the signal emitted by SSEA-1 (coupled to Dylight^{TM} PE). The results indicate that 37.4% of single cells were positive for SSEA-1. These data taken together, suggested that our procedure resulted in the formation of mES-like cells but that colonies contained a mixture of pluripotent and differentiated cells.

To isolate a pure population of pluripotent cells we submitted the cells to flow cytometry coupled to cell sorting. Prior to flow cytometry cells were detached by trypsinization and kept in cold sorting solution (HSBS buffer without calcium or magnesium supplemented with HEPES, 2%FBS and glucose) until analysis. The results indicated that 25% of total event were positive for the SSEA-1 marker (37.4% of singlets) (Figure 6). Those positive cells were sorted and captured in mES media to then be seeded over a fresh layer of feeder cells conditioned in mES media.

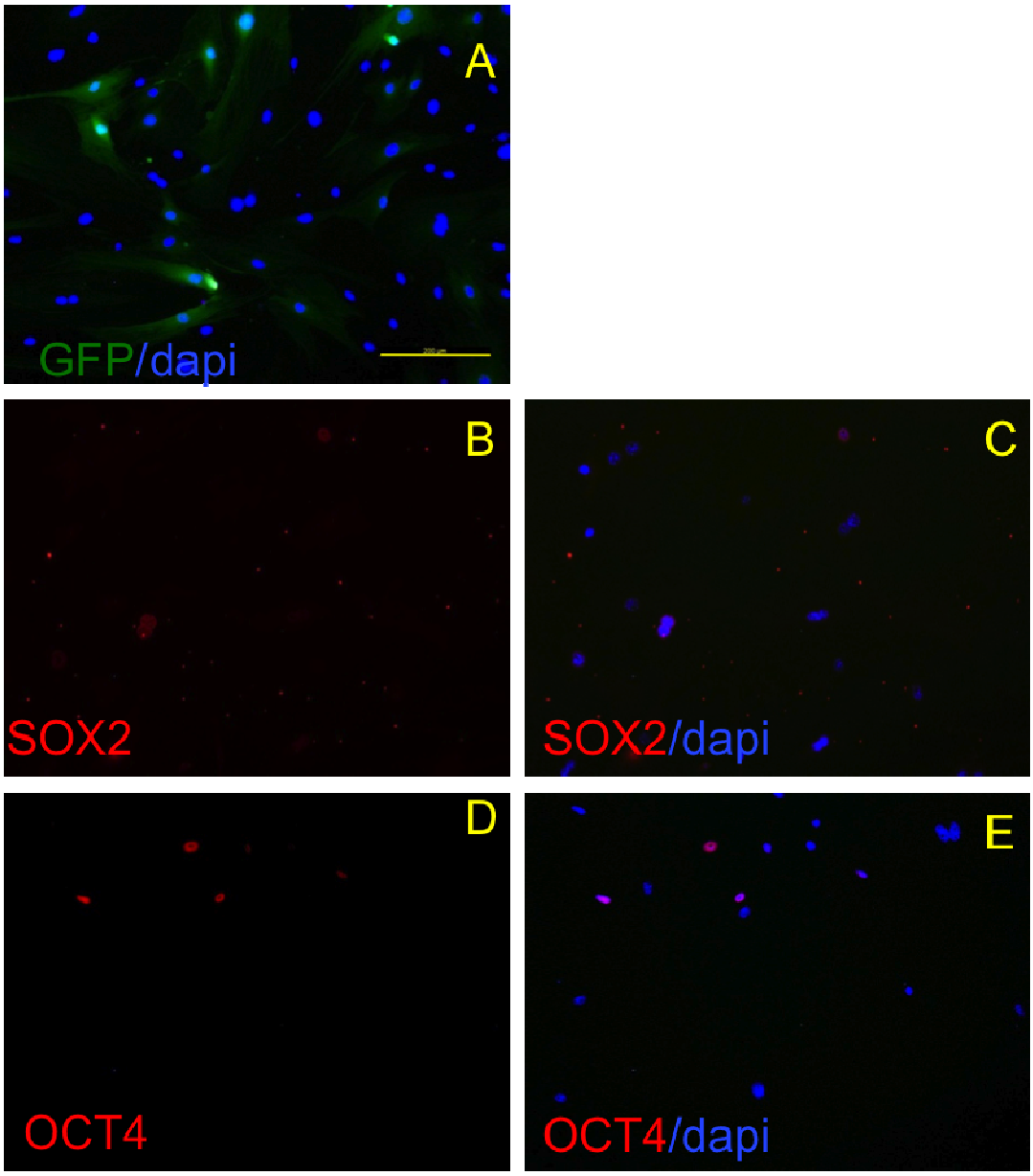
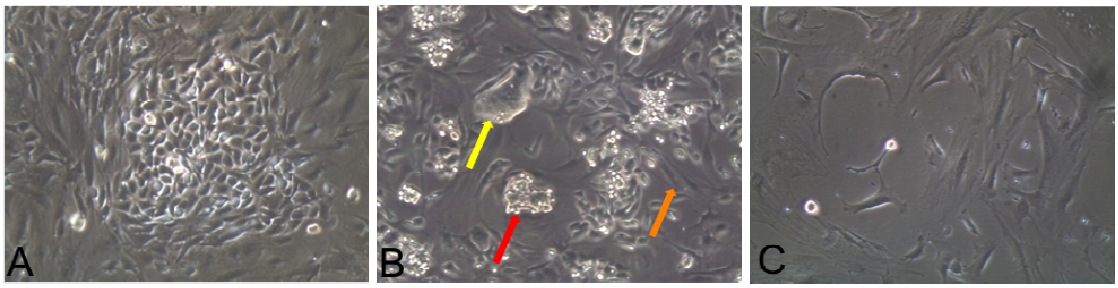
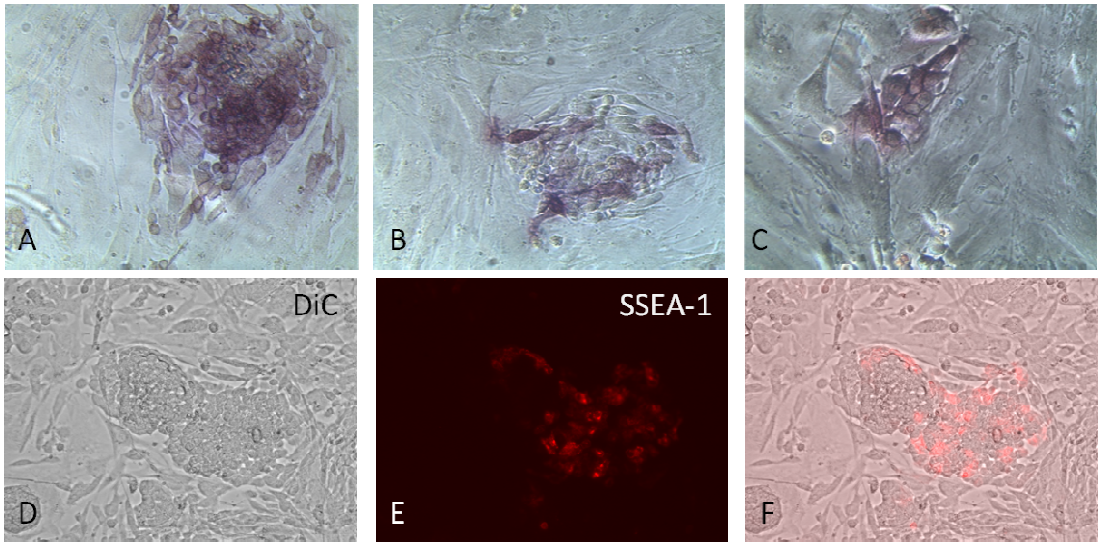


Figure 3. **Efficiency of viral transduction.** Cells were transduced with a GFP-coding retrovirus and immunostained for the ectopic expression of Sox-2 and Oct-4. After 48 hours post transduction the amount of GFP positive fibroblasts was close to 25 % (A). After replate the fibroblasts we evaluated the number of cells overexpressing Sox-2 (B-C) and Oct-4 (D-E). In both cases we observed a similar percentage of cells expressing those markers, 22% and 27% respectively. Bar: 100 nm.



e transduced with
s we observed the
l we were able to
arrow) between
ng KO fibroblasts
id not show any of
(C). Magnification:



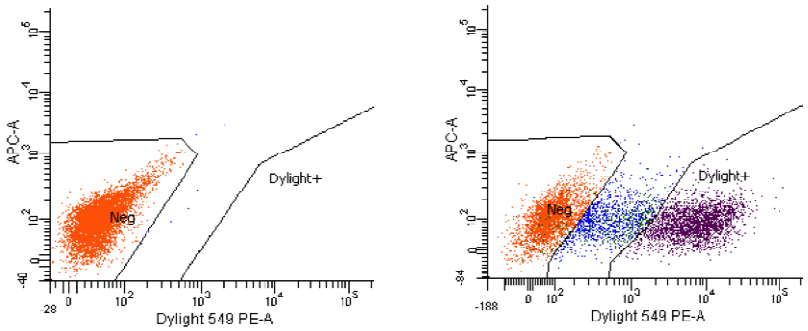
n-pluripotent
r pluripotency
t all cells were
nomenon was
SSEA-1 by

III.4. Characterization of putative Induced Pluripotent Stem Cells

After one day in culture, sorted cells formed colonies similar in shape (Figure7A). In addition, cells forming the new colonies were all positive for the AP assay (fig 7B), in contrast to cells observed prior to sorting. To further establish if the new colonies were induced pluripotent stem cells we ran a series of immunostaining assays using specific markers for pluripotency like Oct-4, Sox-2 and SSEA-1. The studies were done in parallel using our iPS cells generated from Prnp KO mice and commercially available mouse iPS which have been extensively characterized for pluripotency [35]. Our results show that virtually all the cells forming the clusters were positive for those markers but not the cells surrounding the colonies, which are irradiated MEFs, used as a feeder layer. These data, taken together, strongly suggest that the sorted cells were IPS cells. To confirm this hypothesis we performed two other assays for pluripotency: embryoid body formation and teratoma formation assay.

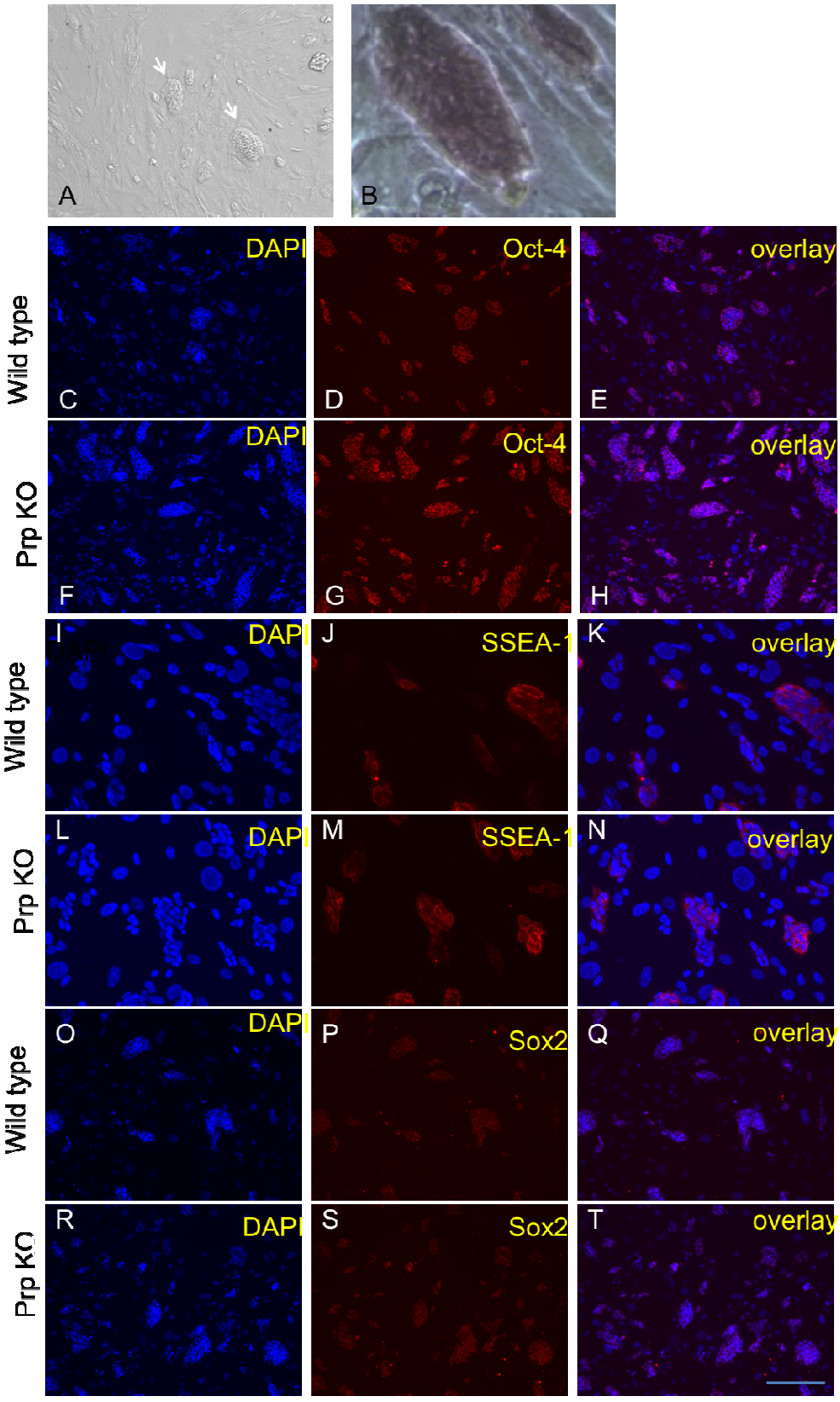
Embryoid bodies (EB) are sac-like structures developed in vitro from embryonic stem cells containing cells belonging to ectodermal, mesodermal and endodermal lineages [70]. We submitted the putative IPS cells (sorted and cultured population of cells) to two types of EB assay: in low-adhesion plates and in microwells as described in material and methods. For both assays we passaged both the putative IPS cells colonies as well as control IPS cells three times in gelatin-coated plates to eliminate iMEFs. Cells were then cultured in suspension in a low-attachment plate in basic media and incubated under standard conditions for 2 days without disturbances. In the alternative protocol, the same density of cells was seeded over a grid of microwells coupled to a cell culture plate (Aggrewell, Stemcell technologies). After 2 days in culture, cells in low-attachment plates showed adherence between them and grew in clusters (not shown). Cells in microwells grew at as clusters at the bottom of the wells (fig 8A, 8C). They were detached from the bottom of the wells by carefully blowing media and then transferred to a low-attachment plate were they kept growing as spherical structures. After 10 days in culture, both systems gave rise to EB, with the main

difference between them being that the microwells gave rise to more uniform structures in size and shape. Starting at 11 days in culture we observed the presence of contractile cells near the pole of the EBs. This suggests that KO iPS cells are able to differentiate into cells of mesoderm origin, supporting the pluripotency capability of the newly generated IPS cells from KO fibroblasts.. Other authors also have observed the appearance of contractile cells in EBs. They identified those cells as cardiomyocites [70-72]. No differences in morphology (figure 8 A-D) were observed between our KO iPS and commercially obtained wt IPS cells.



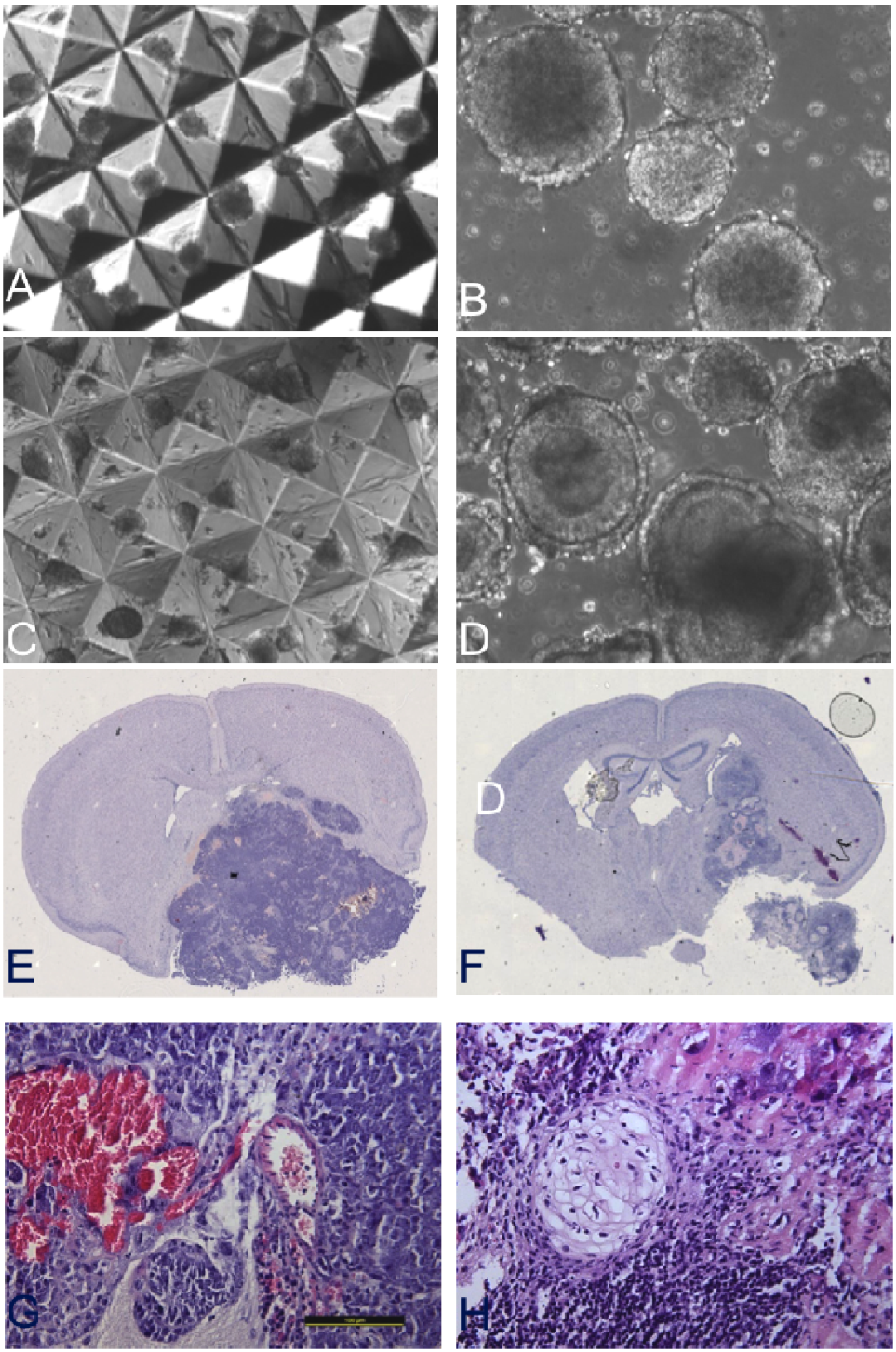
ained with
FACSDiva
rbance for
tted by the
e negative
spectrum
t+, purple
s.

SSEA-1 positive
feeder cells (A).
), a marker for
Sox-2 (O-T) was
ld type bona-fide


A
B
Wild type
DAPI
Oct-4
overlay
C
D
E
Prp KO
DAPI
Oct-4
overlay
F
G
H
Wild type
I
DAPI
J
SSEA-1
K
overlay
Prp KO
L
DAPI
M
SSEA-1
N
overlay
Wild type
O
DAPI
P
Sox2
Q
overlay
Prp KO
R
DAPI
S
Sox2
T
overlay

A second assay to verify the pluripotency capability of the newly generated IPS was a teratoma formation assay. For that we intracranially injected 2x 10^5 cells in mice in the hippocampal area of the right hemisphere. One animal died after three weeks of a severe brain tumor. Surviving animals were sacrificed after 4 weeks post injection and brain tissue was analyzed. All the animals (5/5) showed tumors, suggesting that the KO IPS cells are able to differentiate and form solid tumors. Figure 8E-H shows the histopatological profile of the tumors generated in wt animals. Figure 8 G-H shows details of the structures found in the brain of KO iPS-injected animals as vascular tissue and cartilage. The proportion of animals developing tumors and the morphological characteristic of the tumors were very similar when the experiments were done with both our KO IPS and the well characterized wt IPS cells.

Figure 8 (next page). **Pluripotency assays**. Embryoid body formation assay in was induced by incubating the cells in microwells. Panel A shows the microwells seeded with KO-IPS after 48 hours of plating while panel C shows the WT-IPS at the same point time (200x magnification). Then, spheres were transfer to a low-attachment plate where they grew in suspension. After 7 days cells formed bigger spheres as shown in panel B for KO-IPS and panel D for WT-IPS (100x magnification). For the teratoma formation assay KO-IPS and WT-IPS cells were injected into the brain of wild type animals. At 3 weeks post implantation animals were sacrificed and their brain fixed for H&E staining. Both sets of cells formed solid tumors. Panel E shows a mosaic of pictures taken a 5x magnification of a brain injected with KO-IPS while panel F shows a brain injected with WT-IPS. Panel E and H show a detail of structures found in KO-IPS injected brain. We observed that the lesion present hypervascularization (G) and the development of other structures with morphology similar to cartilage (H). Scale bar: 100 µm.

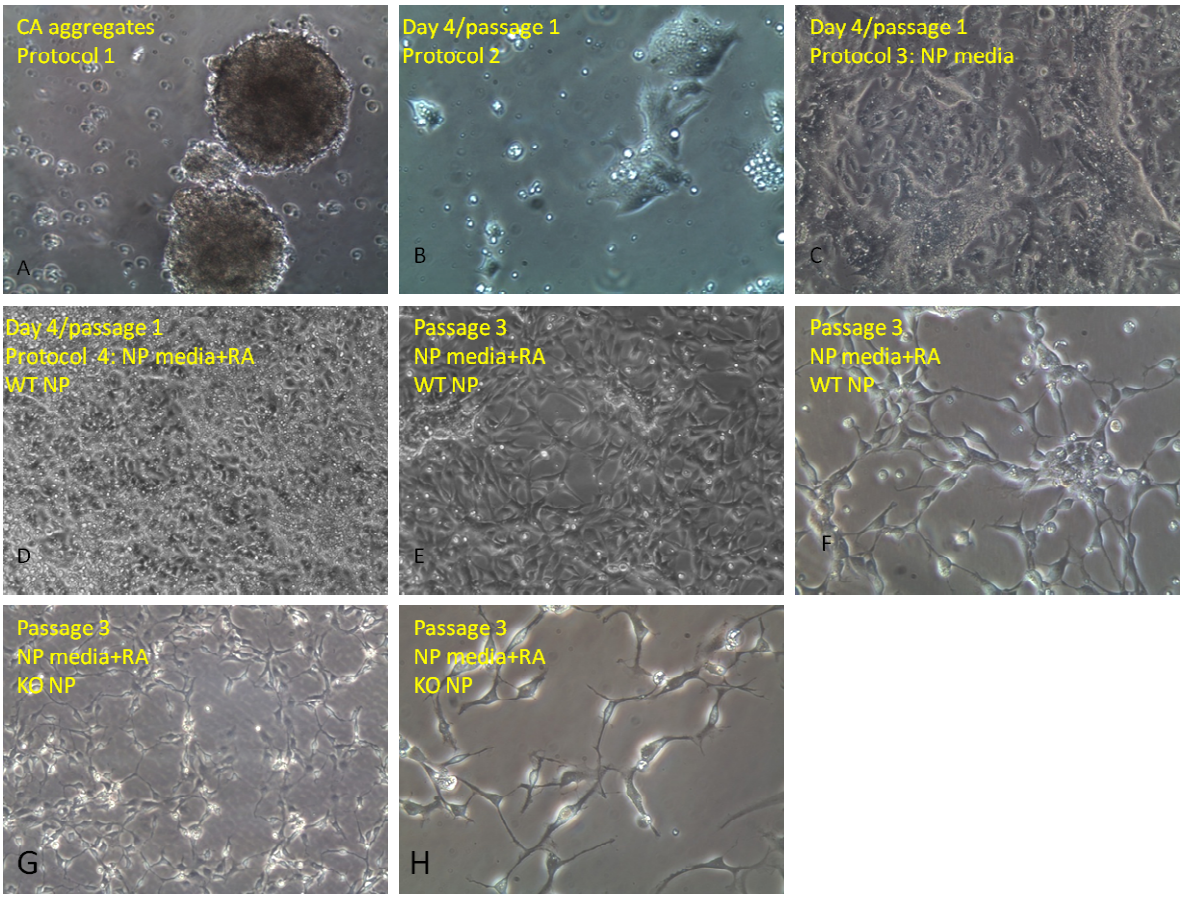

A
B
C
D
E
D
F
G
H

III.5. Differentiation of IPS cells into neural precursors

The next step after obtaining IPS cells from KO fibroblast was to differentiate them into neural precursors. We tried three published protocols of differentiation to evaluate which one resulted in the most homogeneous long lasting population of NPs. The first protocol applied was the one published in 2007 by Bibel *et al* [64]. Briefly, IPS cells are culture as adhesion free-cells in DMEM supplemented with FBS, glutamine, non-essential amino acids and RA. After a few days we observed that cells formed cellular aggregates (CA) in suspension (Figure 9A) that when were cultured over gelatin-coated plates formed heterogeneous populations of cells judged by their morphology (not shown). Since we were looking for homogeneous populations we discarded this protocol. The second approach was based on the article published by Smith *et al*. in 2005 [65]. In contrast to the previous approach, the cells were grown as adherent monolayer over gelatin-coated dishes in a serum-free medium constituted by a mix of DMEM/F-12 and neurobasal media complemented with B27 and a cocktail of apotransferrin, putrescine, insulin, selenite and progesterone. After a few days in culture cells detached from the bottom of the plate giving an extremely low yield of putative neural precursor cells (Figure 9B). We estimated a reduction in the number of cells of 95%. The third protocol applied was that published by Pollard *et al* in 2006 [66]. As in the previous protocol cells were cultured over a gelatin-coated surface in the presence of NP media (DMEM/F12/Basal media plus B27 and N2 supplements and human apotransferrin, Fibroblast Growth Factor (FGF), and Epidermal Growth Factor (EGF)). Cells showed better adherence to the bottom of the plate only when cultured at high concentrations (Figure 9C); at lower cell density cells died and detached. We tried to optimize this procedure by adding RA (which is known to induce differentiation toward neuronal lineage and improve cell survival [73]) and leaving the cells to reach over confluence prior to switch the media from mES to NP media. Upon changing the media, we observed a massive detachment of dead cells that were removed by washing the plate twice with PBS containing calcium and magnesium after each daily media change. However, we also observed constant proliferation of new adherent cells (Figure 9D); Then, we implemented another protocol (personal

finding, not published) which combine the use of growth factors and RA. We plated the pluripotent cells in at $3x10^5$ cells/cm^2 in NP supplemented with 5 µm RA. Next day media was changed for the same fresh solution. At day 3 and 4 media was removed and replaced with NP supplemented with 3 µm RA, and finally on day 5 and 6 media was removed and replaced with NP media supplemented with 3 µm RA. We also observed a high rate of apoptotic detaching cells and proliferation of the adherent layer. The final population was passaged by trypsinization (0.05% trypsin/EDTA) at day 6 or 7 over a gelatin-coated plate and then the media was changed every other day (NP media supplemented with 1 µm RA). After 2 passages cells showed a triangular morphology in the soma and with short projections interlocking the cells. Cells with similar morphology are found after the differentiation process of mouse ES cells as describes in [66]. The authors described those cells as similar to radial glia cells capable of generating neurons, astrocytes, and oligodendrocytes.

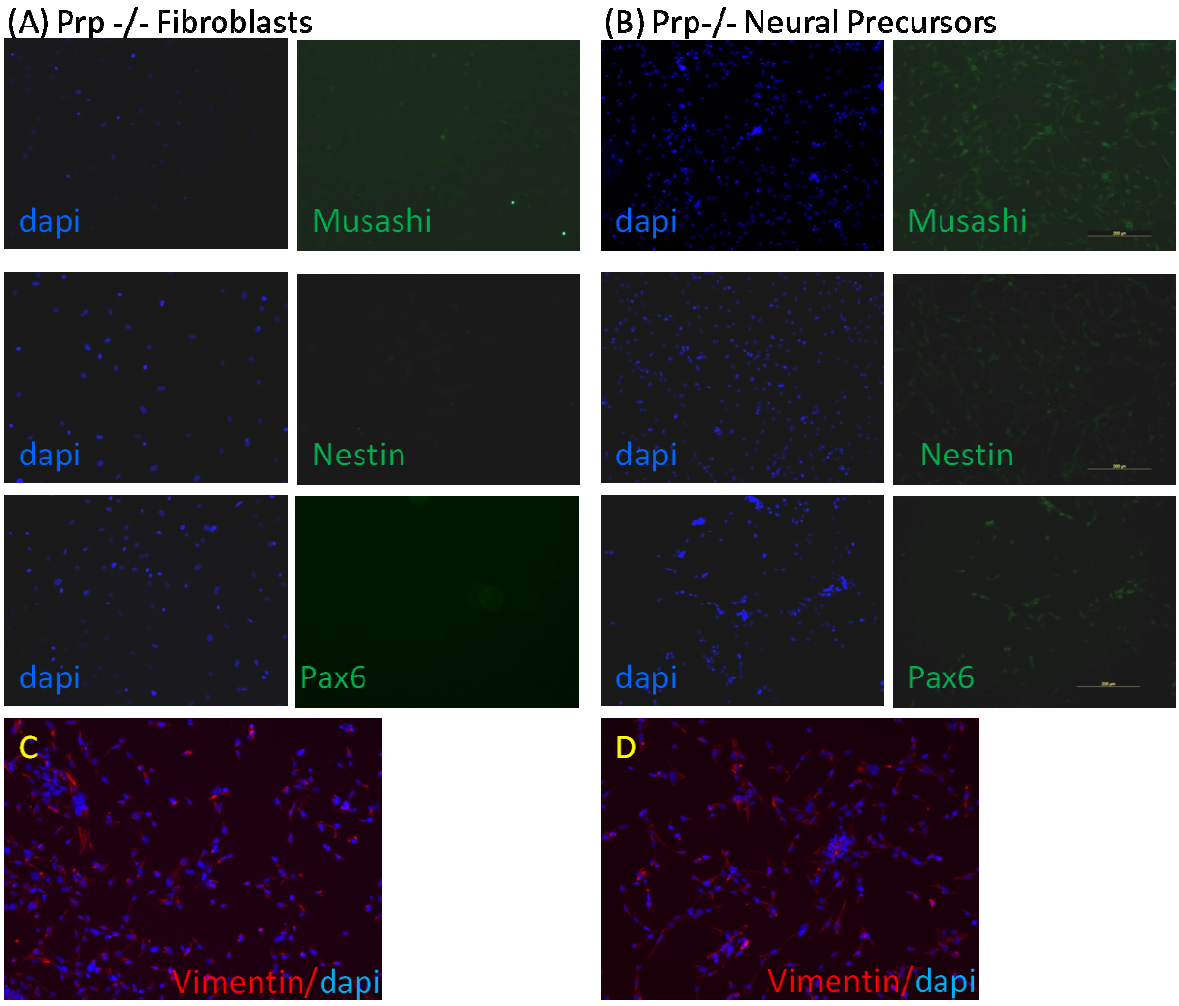
We observed the same outcome, both in terms of morphology and yield, when started with KO IPS or wt IPS cells (fig 9E-H), suggesting that KO IPS cells behave like wt cells in terms of neuronal progenitor development. It is important to mention that KO-NP cells were intracranially injected in wild type animals and we did not observe any evidence of brain tumors at least 6 weeks after implantation. This indicates that the differentiated population of cells losses the tumorigenic potential.



ls. WT IPS cells Bs (A) that when ions (not shown). growth factors we nistration of bFGF etinoic acid to the aintaining a radial- ion as wt cells (G-

III.6. Characterization of Neural Precursors cells.

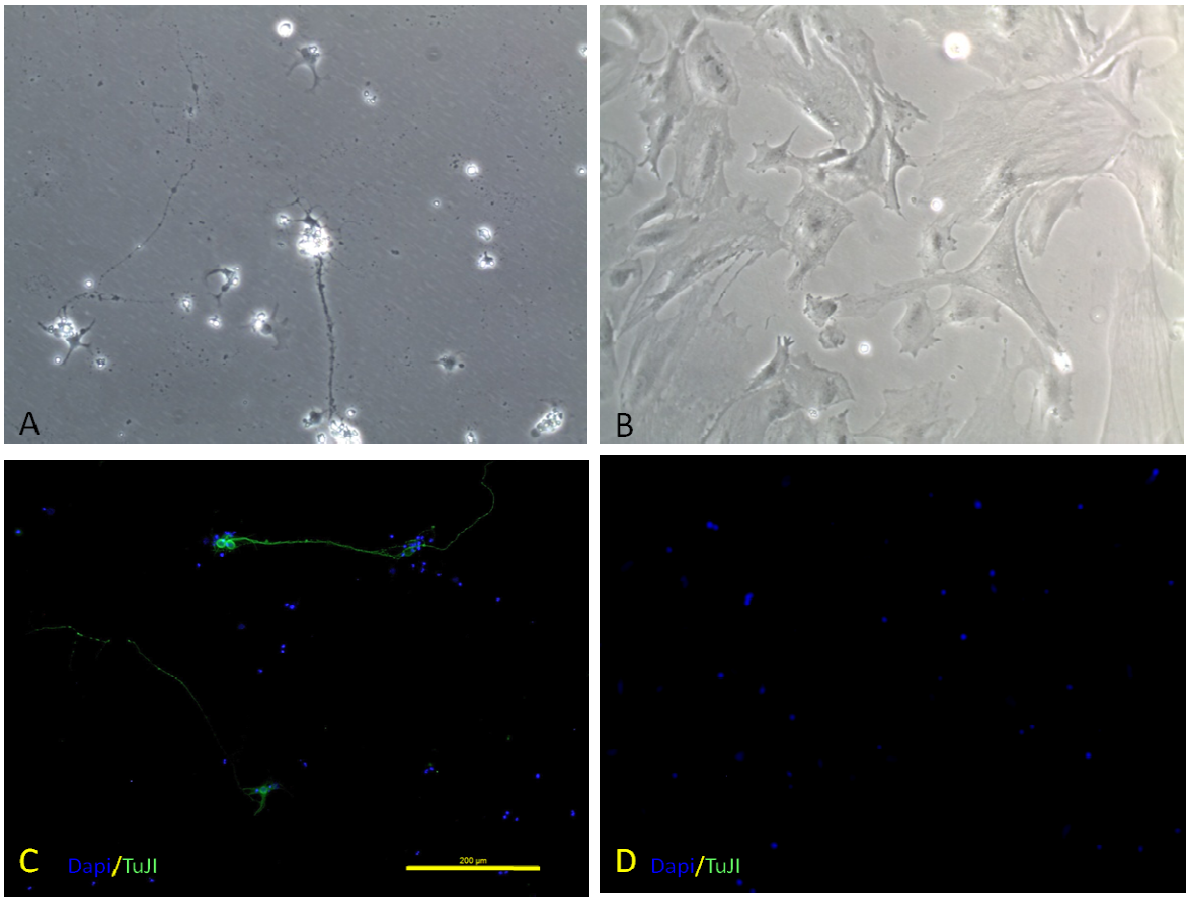
To further characterize that the population of cells generated by incubation in media containing EGF, FGF and RA consist of NP cells, we tested if the cells express markers for neural precursor cells.



KO fibroblasts
nemistry. KO-
We observed
r neural stem
ells, D, Wt-NP

We compared the expression of those markers against the basal expression in fibroblasts and we observed the up regulation of Nestin, Musashi and Pax6 (fig 10 A-B). Also, we observed an upregulation of the marker for neuronal stem cells Vimentin in both KO NP and wt NP cells relative to the IPS cells (not shown).

To further characterize the developmental properties of NP cells we induced their differentiation into neurons and glial cells. For neuronal differentiation we seeded the cells over an ornithine/laminin treated dish in the same basic formula for NP differentiation, but lacking the growth factors. Although most of the cells died, after 10 days we observed rounded cells and long projections resembling pyramidal neurons (fig 11A). Also, we found that those cells were positive for the neuron specific TuJI antibody (fig 11C). For glial differentiation NP cells were seeded over a gelatin treated plate in the presence of NP media lacking growth factors but supplemented with 2% FBS. Under this condition cells survived and grew into flat and expanded cells. Some of those showed a protuberant nucleus at the center of the cell (fig 11B). Although the morphology resembles glial cells we need to perform further analysis to confirm the glial-like properties of the resulting population. Taken together this data suggest that the initial KO NP population of cells generated from skin-derived IPS cells have the intrinsic capability to differentiate into neurons.



rogenitors cells.
r cells showed a
essed markers for
a showed a glial
D). Bar: 200 µm

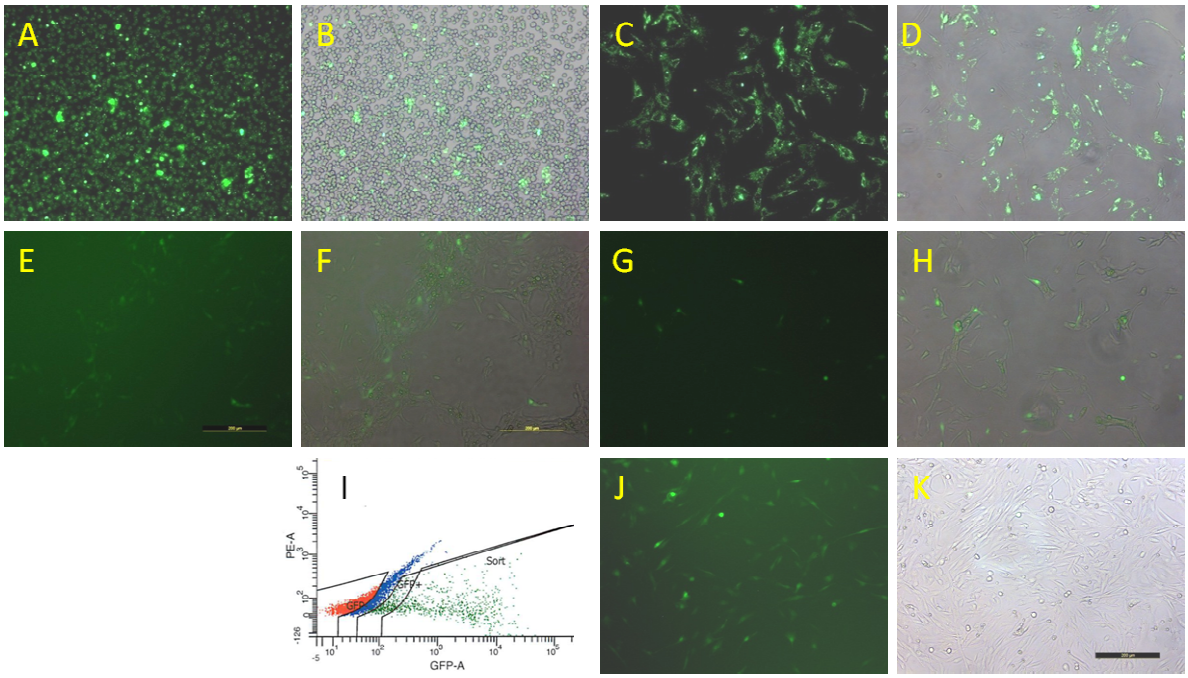
III.7. Generation of traceable NP cells

In order to track the NP cells once implanted into the brain of experimental animals we explored three different approaches: cell labeling with fluorescent lipophilic tracers, transfection with GFP coding vectors and infection with GFP-coding retrovirus.

The first approach was implemented by treating the NP cells with SP-$DiOC_{18}$(3), a fluorolipophilic dye which is able to diffuse laterally within cellular

membranes. After incubation of the cells with SP-$DiOC_{18}$(3) for 4 min at 37°C followed for 15 min at 4°C we observed that the lipophilic dye effectively marked the cells as seen by microscopy (fig 12A-B). Those cells were reseeded in a gelatin-coated plate containing glial media to evaluate the survival after staining. Even after 12 days of culture we observed that cells survived and retained the marker along the plasma membrane and internal compartments (fig 12C-D).

The second approach was to transfect the cells with a GFP-coding plasmid using lipofectamine. The initial result indicated a transfection rate of 50%; however the transfected cells died soon after two passages (data not shown). A second attempt to make the cells express GFP was to transduce them with a GFP coding retrovirus. For that we transduced platinum E cells (NIH-3T3 derived cells) with the pMXs-IRES-GFP vector to induce the production of GFP-coding retrovirus. We then concentrated the viruses by centrifugation to finally resuspend them in NP media. We tested the efficiency of transduction in wild type NH3 fibroblasts obtaining efficiency close to 100% when evaluated by microscopy (data not shown). Using the same batch of viruses we infected KO-NP cultures. After 24 hours we replaced the virus-containing media with fresh NP media and evaluated the presence of green NP cells. We observed expression of GFP in some cells after 48 hours post-infection (fig 12E-F). We expanded the culture, however after two passages the fraction of positive cells was lower than expected. To enrich the GFP (+) population we submitted the cells to flow cytometry followed by cell sorting by using the FACSDiva system (fig 12 I). A pure population of GFP-positive cells was collected and then cultured under standard conditions for NP cells. We observed that after few days GFP (+) cells present in the culture counted for only a small percentage of the total population. This result suggested that the expression of GFP is silenced in NP cells or that the accumulation of GFP might be cytotoxic.



NP cells after
scent dye (A-
C-D) showing
GFP-coding
duction (E-F).
ng and GFP-
opulation was
ve dilution of

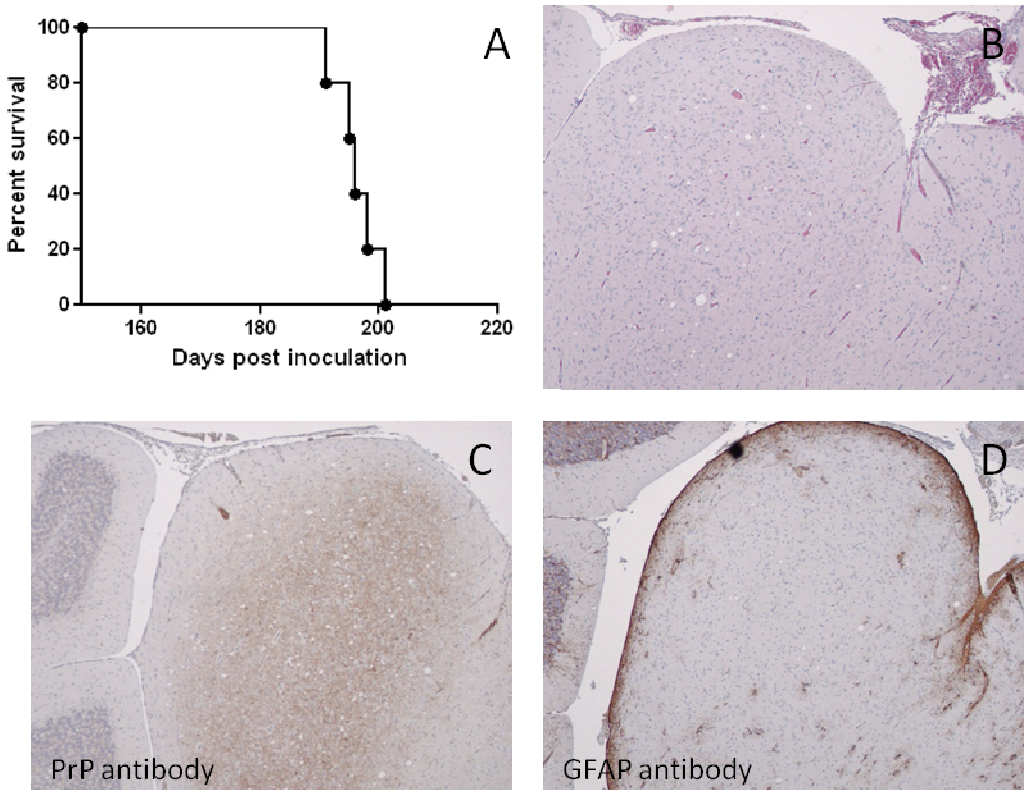
III.8. Future Plans

In order to test the working hypothesis we need to implant the neural precursors derived from KO PS cells, as well as their wild type counterpart, into animals infected with prions to the evaluate if they perform better in a series of tests to evaluate the clinical symptoms, survival rate, neuropathological analysis of the brain and biochemical parameters. To set up the conditions for testing the effect of NP implantation in animals infected with prions, we performed a detailed characterization of this animal model. Prions were injected i.p. in wild type mice. The most important parameter to analyze is the survival time of the treated animals

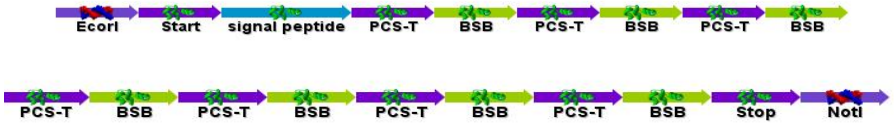
(Fig. 13A). We expect to see an increased survival of the animals receiving the treatment, which should be dependent on the amount of cells implanted. Furthermore, we will evaluate the performance of the animals in the rotarod test, which measures motor coordination. Also, animals will be tested in an open field assay to measure general physical activity and response to a new environment. For neuropathological analysis, half of the brain will be fixed in paraformaldehyde for H&E staining in order to measure the intensity of brain lesions product of the disease (vacuolation), as well as the pattern where those sings appear (Fig. 13B). Astrogliosis will be evaluated by immunostaining with an anti-GFAP antibody and the accumulation of the prion protein with an anti-prion antibody (fig 13C and D). Figure 13 shows those results for an untreated animal.

In future experiments we would like to increase the efficacy of the stem cell treatment by using engineered cells aimed to release BDNF to the microenvironment. Currently, we are creating stable lines of NP cells transfected with BNDF. In parallel we are working to create stable lines of NP capable of releasing beta-sheet breaker peptides (BSBs), which have been shown to inhibit and reverse prion replication in vitro and in vivo [28] We designed a gene called “Beta-sheet breaker Super Gene” which include an exportation signal followed by BSB peptides linked by thrombin-cleavage segments (PCS-T). The BSB sequences are based in data from previous publications where the authors showed the effects of peptides on the blockage of fibrillation of beta-amyloid and prion peptides [28, 30]. We expect that the cells will translate the peptide and then it could be transported to the extracellular space. Once outside the cells the arg-peptidase thrombin can cut the peptide to release several fragments of BSB. Diagram 14 depicts the design of the gene. We chose the PCS-T linker since thrombin is known to be secreted by brain endothelial cells [74] and thrombin is an activator of astrogliosis [75, 76], which strongly suggest that thrombin is present in the brain parenchyma of prion infected animals. Moreover, the cell implantation surgery will open wounds disrupting the brain-blood barrier, allowing the uptake of thrombin in the brain.

Before implantation we should determine if: 1) the complete BSB peptide will be externalized by the NP cells, 2) if the thrombin is able to catalyze the cleavage of the peptide, and 3) if the peptides are effective in vitro. To analyze the expression of BSBs we produced an antibody specific against prion and beta-amyloid which can be used in ELISA assays. If this approach fails, we will clone the BSB gene in tandem with a DKK tag to then detect the tag with an anti-DKK antibody. Once we are sure we have expression of BSBs, the peptide will be digested by thrombin in vitro and then the molecular weight of the resultant peptides will be analyzed by western blot. 4) Finally, we will test if the BSB peptides are as effective when they are flanked by the residuals amino acids of the PCS-T linker as when they are not.



. Mice show clear
coordination after
prion protein. They
e disease. Graph A
hey show extended
) as well deposition



BSB prion after digestion: GSDAAPAGAPVLVP

Diagram 14. **Structure of the beta-sheet breaker super gene.** We expect that seven segments containing BSB (green) can be released after cleavage into the extracellular space. PCS-T (purple) sequences are consensus sequences for thrombin, a peptidase secreted by brain endothelial cells.

IV. Conclusions

The use of stem cells for human cell therapies opens a new window of hope for regenerative medicine for individuals affected with a wide range of diseases like neurodegenerative diseases, myocardial failure, stroke, traumatic brain injury and many other others disorders.

Embryonic stem cells form part of the blastula and are able to differentiate to all types of cells in the body. In vitro, ESC can be extracted and maintained in vitro for an unlimited number of passages while maintaining their pluripotent capabilities. Furthermore, ESC ban be differentiated in vitro to different cellular populations, including adult stem cells like neural precursors or fully differentiated cells like neurons. Neural Precursors have the potential to complete their developmental fate when they are grafted being able to survive and integrate within the host neuronal tissue. These advantages of neuronal stem cells position them as a good biomaterial for the development of replacement therapies in order to regenerate lost brain cells due to aging or neurodegenerative diseases. Besides, neuronal stem cells have the potential to act positively over the host brain by paracrine action

thereby stimulating the survival of existing neuronal cells, stimulation of neurogenesis and synaptogenesis. To date many groups have shown the potential of stem cells, however their use in clinical practice has been narrow due to the limited source of human embryonic stem cells. Overcoming those obstacles, IPS cell technology offers the possibility to generate ESC-like cells from somatic cells like skin or blood cells. This offers the possibility to generate stem cells from adult patients to then use them for regenerative therapies within the same patient without the threat of immune rejection. Besides, thanks to the advances in molecular biology, IPS cells could be treated to repair genetic defects and reinserted into the same patient. Although the technology is promising, it must still be improved in order to be safe in the clinical practice.

In this work we proposed to investigate if IPS cells could be generated from animals resistant to prion diseases. We generated IPS cells from transgenic mice lacking the prion protein, which we know is resistant to the toxic damage of prion infection. Skin cells from those animals were culture in vitro and then transduced to overexpress Oct-4, Sox-2, Myc-C and Klf-4. Transduced cells showed to be different in morphology and developmental capabilities. They expressed pluripotency markers and were capable to form solid tumors and embryoid bodies. In vitro those cells were differentiated to neural precursor cells that were able to give raise to neurons in vitro. In preparation for the implantation of the neuronal precursor cells in the brain of sick animals, we tested several protocols to trace the cells by fluorescence. It is important to note that IPS cells were generated from the skin of old animals (1 year old mice), indicating that the age of the animals is not a limiting factor for reprogramming of prp-/- cells.

Next experiments include engrafting the neural precursors devoid of the prion protein as well as wild type neural precursors in the experimental animals and evaluating if the treatment extends the lifespan. In parallel, other experiments to do (work which is currently in progress) include to engineering the cells to make them release beta-sheet breakers and BDNF to induce the elimination of misfolded prions

and to improve the survival and functional capabilities of the existing network, respectively.

We expect that our studies could serve as proof of concept of the use of neural precursors generated from fibroblasts-derived pluripotent cells of prion affected patients. In theory, those cells could be engineered in the test tube to decrease the expression of prion protein and be more resistant once engrafted in the brain or peripheral tissue of the patient.

V. Bibliography

1. Hebert LE, Scherr PA, Bienias JL, Bennett DA, Evans DA. Alzheimer disease in the US population: prevalence estimates using the 2000 census. *Arch Neurol* 2003, 60(8):1119-1122.
2. Center for Disease Control and Prevention. *Alzheimer's disease Statistics* accessed by Jan 2012(accessed by Jan 2012).
3. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006, 126(4):663-676.
4. Yahata N, Asai M, Kitaoka S, Takahashi K, Asaka I, Hioki H, Kaneko T, Maruyama K, Saido TC, Nakahata T, Asada T, Yamanaka S, Iwata N, Inoue H. Anti-Abeta drug screening platform using human iPS cell-derived neurons for the treatment of Alzheimer's disease. *PLoS One* 2011, 6(9):e25788.
5. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. *Nat Rev Neurosci* 2003, 4(1):49-60.
6. Palop JJ, Mucke L. Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nat Neurosci* 2010, 13(7):812-818.
7. Doyle KM, Kennedy D, Gorman AM, Gupta S, Healy SJ, Samali A. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. *J Cell Mol Med* 2011, 15(10):2025-2039.
8. Stella R, Massimino ML, Sandri M, Sorgato MC, Bertoli A. Cellular prion protein promotes regeneration of adult muscle tissue. *Mol Cell Biol* 2010, 30(20):4864-4876.
9. Lasmezas CI. Putative functions of PrP(C). *Br Med Bull* 2003, 66:61-70.
10. Klamt F, Dal-Pizzol F, Conte da Frota ML, Jr., Walz R, Andrades ME, da Silva EG, Brentani RR, Izquierdo I, Fonseca Moreira JC. Imbalance of antioxidant defense in mice lacking cellular prion protein. *Free Radic Biol Med* 2001, 30(10):1137-1144.

11. Chiarini LB, Freitas AR, Zanata SM, Brentani RR, Martins VR, Linden R. Cellular prion protein transduces neuroprotective signals. *Embo J* 2002, 21(13):3317-3326.
12. Mallucci GR, Ratte S, Asante EA, Linehan J, Gowland I, Jefferys JG, Collinge J. Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *Embo J* 2002, 21(3):202-210.
13. Bueler H, Raeber A, Sailer A, Fischer M, Aguzzi A, Weissmann C. High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. *Mol Med* 1994, 1(1):19-30.
14. Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, Brandner S, Aguzzi A, Weissmann C. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *Embo J* 1996, 15(6):1255-1264.
15. Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A. Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature* 1996, 379(6563):339-343.
16. Chesebro B, Trifilo M, Race R, Meade-White K, Teng C, LaCasse R, Raymond L, Favara C, Baron G, Priola S, Caughey B, Masliah E, Oldstone M. Anchorless prion protein results in infectious amyloid disease without clinical scrapie. *Science* 2005, 308(5727):1435-1439.
17. McNally KL, Ward AE, Priola SA. Cells expressing anchorless prion protein are resistant to scrapie infection. *J Virol* 2009, 83(9):4469-4475.
18. Brundin P, Melki R, Kopito R. Prion-like transmission of protein aggregates in neurodegenerative diseases. *Nat Rev Mol Cell Biol* 2010, 11(4):301-307.
19. Meyer-Luehmann M, Coomaraswamy J, Bolmont T, Kaeser S, Schaefer C, Kilger E, Neuenschwander A, Abramowski D, Frey P, Jaton AL, Vigouret JM, Paganetti P, Walsh DM, Mathews PM, Ghiso J, Staufenbiel M, Walker LC, Jucker M. Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host. *Science* 2006, 313(5794):1781-1784.

20. Eisele YS, Obermuller U, Heilbronner G, Baumann F, Kaeser SA, Wolburg H, Walker LC, Staufenbiel M, Heikenwalder M, Jucker M. Peripherally applied Abeta-containing inoculates induce cerebral beta-amyloidosis. *Science* 2010, 330(6006):980-982.
21. Morales R, Duran-Aniotz C, Castilla J, Estrada LD, Soto C. De novo induction of amyloid-beta deposition in vivo. *Mol Psychiatry* 2011.
22. Munch C, O'Brien J, Bertolotti A. Prion-like propagation of mutant superoxide dismutase-1 misfolding in neuronal cells. *Proc Natl Acad Sci U S A* 2011, 108(9):3548-3553.
23. Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med* 2008, 14(5):504-506.
24. Kordower JH, Chu Y, Hauser RA, Olanow CW, Freeman TB. Transplanted dopaminergic neurons develop PD pathologic changes: a second case report. *Mov Disord* 2008, 23(16):2303-2306.
25. Li JY, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, Lashley T, Quinn NP, Rehncrona S, Bjorklund A, Widner H, Revesz T, Lindvall O, Brundin P. Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nat Med* 2008, 14(5):501-503.
26. Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E, Crews L, Spencer B, Masliah E, Lee SJ. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proc Natl Acad Sci U S A* 2009, 106(31):13010-13015.
27. Sigurdsson EM, Permanne B, Soto C, Wisniewski T, Frangione B. In vivo reversal of amyloid-beta lesions in rat brain. *J Neuropathol Exp Neurol* 2000, 59(1):11-17.
28. Soto C, Kascsak RJ, Saborio GP, Aucouturier P, Wisniewski T, Prelli F, Kascsak R, Mendez E, Harris DA, Ironside J, Tagliavini F, Carp RI, Frangione B. Reversion of prion protein conformational changes by synthetic beta-sheet breaker peptides. *Lancet* 2000, 355(9199):192-197.

29. Poduslo JF, Curran GL, Kumar A, Frangione B, Soto C. Beta-sheet breaker peptide inhibitor of Alzheimer's amyloidogenesis with increased blood-brain barrier permeability and resistance to proteolytic degradation in plasma. *J Neurobiol* 1999, 39(3):371-382.
30. Soto C, Kindy MS, Baumann M, Frangione B. Inhibition of Alzheimer's amyloidosis by peptides that prevent beta-sheet conformation. *Biochem Biophys Res Commun* 1996, 226(3):672-680.
31. Briggs R, King TJ. Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. *Proc Natl Acad Sci U S A* 1952, 38(5):455-463.
32. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997, 385(6619):810-813.
33. Ogonuki N, Inoue K, Yamamoto Y, Noguchi Y, Tanemura K, Suzuki O, Nakayama H, Doi K, Ohtomo Y, Satoh M, Nishida A, Ogura A. Early death of mice cloned from somatic cells. *Nat Genet* 2002, 30(3):253-254.
34. Ogura A, Inoue K, Ogonuki N, Lee J, Kohda T, Ishino F. Phenotypic effects of somatic cell cloning in the mouse. *Cloning Stem Cells* 2002, 4(4):397-405.
35. Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007, 448(7151):318-324.
36. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* 2009, 27(3):275-280.
37. Dimos JT, Rodolfa KT, Niakan KK, Weisenthal LM, Mitsumoto H, Chung W, Croft GF, Saphier G, Leibel R, Goland R, Wichterle H, Henderson CE, Eggan K. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 2008, 321(5893):1218-1221.
38. Ebert AD, Yu J, Rose FF, Jr., Mattis VB, Lorson CL, Thomson JA, Svendsen CN. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 2009, 457(7227):277-280.

39. Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, Hargus G, Blak A, Cooper O, Mitalipova M, Isacson O, Jaenisch R. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 2009, 136(5):964-977.
40. Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, Sasai Y, Takahashi M. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. *J Cell Sci* 2009, 122(Pt 17):3169-3179.
41. Ishikawa T, Hagiwara K, Ochiya T. Generation and hepatic differentiation of human iPS cells. *Methods Mol Biol* 2012, 826:103-114.
42. Choi KD, Yu J, Smuga-Otto K, Salvagiotto G, Rehrauer W, Vodyanik M, Thomson J, Slukvin I. Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells* 2009, 27(3):559-567.
43. Taura D, Noguchi M, Sone M, Hosoda K, Mori E, Okada Y, Takahashi K, Homma K, Oyamada N, Inuzuka M, Sonoyama T, Ebihara K, Tamura N, Itoh H, Suemori H, Nakatsuji N, Okano H, Yamanaka S, Nakao K. Adipogenic differentiation of human induced pluripotent stem cells: comparison with that of human embryonic stem cells. *FEBS Lett* 2009, 583(6):1029-1033.
44. Kim J, Efe JA, Zhu S, Talantova M, Yuan X, Wang S, Lipton SA, Zhang K, Ding S. Direct reprogramming of mouse fibroblasts to neural progenitors. *Proc Natl Acad Sci U S A* 2011, 108(19):7838-7843.
45. Qiang L, Fujita R, Yamashita T, Angulo S, Rhinn H, Rhee D, Doege C, Chau L, Aubry L, Vanti WB, Moreno H, Abeliovich A. Directed conversion of Alzheimer's disease patient skin fibroblasts into functional neurons. *Cell* 2011, 146(3):359-371.
46. Lin T, Ambasudhan R, Yuan X, Li W, Hilcove S, Abujarour R, Lin X, Hahm HS, Hao E, Hayek A, Ding S. A chemical platform for improved induction of human iPSCs. *Nat Methods* 2009, 6(11):805-808.
47. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science* 2008, 322(5903):945-949.

48. Woltjen K, Michael IP, Mohseni P, Desai R, Mileikovsky M, Hamalainen R, Cowling R, Wang W, Liu P, Gertsenstein M, Kaji K, Sung HK, Nagy A. piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 2009, 458(7239):766-770.
49. Gonzalez F, Barragan Monasterio M, Tiscornia G, Montserrat Pulido N, Vassena R, Batlle Morera L, Rodriguez Piza I, Izpisua Belmonte JC. Generation of mouse-induced pluripotent stem cells by transient expression of a single nonviral polycistronic vector. *Proc Natl Acad Sci U S A* 2009, 106(22):8918-8922.
50. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008, 322(5903):949-953.
51. Narsinh KH, Jia F, Robbins RC, Kay MA, Longaker MT, Wu JC. Generation of adult human induced pluripotent stem cells using nonviral minicircle DNA vectors. *Nat Protoc* 2011, 6(1):78-88.
52. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, Ko S, Yang E, Cha KY, Lanza R, Kim KS. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 2009, 4(6):472-476.
53. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S. Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 2009, 4(5):381-384.
54. Plews JR, Li J, Jones M, Moore HD, Mason C, Andrews PW, Na J. Activation of pluripotency genes in human fibroblast cells by a novel mRNA based approach. *PLoS One* 2010, 5(12):e14397.
55. Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes Dev* 2010, 24(20):2239-2263.
56. Penn MS, Dong F, Klein S, Mayorga ME. Stem cells for myocardial regeneration. *Clin Pharmacol Ther* 2011, 90(4):499-501.

57. Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res* 2011, 109(8):923-940.
58. Yamasaki TR, Blurton-Jones M, Morrissette DA, Kitazawa M, Oddo S, LaFerla FM. Neural stem cells improve memory in an inducible mouse model of neuronal loss. *J Neurosci* 2007, 27(44):11925-11933.
59. Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Muller FJ, Loring JF, Yamasaki TR, Poon WW, Green KN, LaFerla FM. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci U S A* 2009, 106(32):13594-13599.
60. Relano-Gines A, Lehmann S, Bencsik A, Herva ME, Torres JM, Crozet CA. Stem cell therapy extends incubation and survival time in prion-infected mice in a time window-dependant manner. *J Infect Dis* 2011, 204(7):1038-1045.
61. Takahashi K, Okita K, Nakagawa M, Yamanaka S. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* 2007, 2(12):3081-3089.
62. Stemgent. Mouse IPS cell culture protocol 2011.
63. Meissner A, Wernig M, Jaenisch R. Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat Biotechnol* 2007, 25(10):1177-1181.
64. Bibel M, Richter J, Lacroix E, Barde YA. Generation of a defined and uniform population of CNS progenitors and neurons from mouse embryonic stem cells. *Nat Protoc* 2007, 2(5):1034-1043.
65. Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. *PLoS Biol* 2005, 3(9):e283.
66. Pollard SM, Benchoua A, Lowell S. Neural stem cells, neurons, and glia. *Methods Enzymol* 2006, 418:151-169.
67. Pease S, Braghetta P, Gearing D, Grail D, Williams RL. Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev Biol* 1990, 141(2):344-352.

68. Wada HG, VandenBerg SR, Sussman HH, Grove WE, Herman MM. Characterization of two different alkaline phosphatases in mouse teratoma: partial purification, electrophoretic, and histochemical studies. *Cell* 1976, 9(1):37-44.
69. Fox N, Damjanov I, Martinez-Hernandez A, Knowles BB, Solter D. Immunohistochemical localization of the early embryonic antigen (SSEA-1) in postimplantation mouse embryos and fetal and adult tissues. *Dev Biol* 1981, 83(2):391-398.
70. Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an in vitro model of mouse embryogenesis. *Exp Physiol* 2000, 85(6):645-651.
71. Maltsev VA, Wobus AM, Rohwedel J, Bader M, Hescheler J. Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res* 1994, 75(2):233-244.
72. Kolossov E, Fleischmann BK, Liu Q, Bloch W, Viatchenko-Karpinski S, Manzke O, Ji GJ, Bohlen H, Addicks K, Hescheler J. Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. *J Cell Biol* 1998, 143(7):2045-2056.
73. Sato Y, Heuckeroth RO. Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth in vitro. *Dev Biol* 2008, 320(1):185-198.
74. Yin X, Wright J, Wall T, Grammas P. Brain endothelial cells synthesize neurotoxic thrombin in Alzheimer's disease. *Am J Pathol* 2010, 176(4):1600-1606.
75. Shirakawa H, Sakimoto S, Nakao K, Sugishita A, Konno M, Iida S, Kusano A, Hashimoto E, Nakagawa T, Kaneko S. Transient receptor potential canonical 3 (TRPC3) mediates thrombin-induced astrocyte activation and upregulates its own expression in cortical astrocytes. *J Neurosci* 2010, 30(39):13116-13129.

76. Mhatre M, Nguyen A, Kashani S, Pham T, Adesina A, Grammas P. Thrombin, a mediator of neurotoxicity and memory impairment. *Neurobiol Aging* 2004, 25(6):783-793.

VI. Vita

Dennisse Andrea Gonzalez-Romero was born in Puente Alto, Santiago, Chile on December 13, 1980, daughter of Benedicto Antonio Gonzalez-Karfach and Cecilia Angelica Romero-Gonzalez. After completing her work at Carmela Carvajal de Prat High School, Santiago, Chile in 1998, she entered University of Chile where she received the degree of Bachelor in Biochemistry in 2003 and the "Professional Title" in 2005. For the next two years, she worked as research assistant in the Department of Neurology at The University of Texas Medical Branch at Galveston. In 2007 she joined the Graduate School of Biomedical Sciences at The University of Texas Medical Branch at Galveston. In 2009 she moved to The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences.

Permanent address:
3000 Sage Road, Apt 1227
Houston, Texas 77056