

generation of a neural stem progenitor cell line, transfected with green fluorescent protein for *in VIVO* MONITORING

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**Generación de una línea de progenitores neurales (NPSCs), transfectadas con el gen de la proteína fluorescente verde (GFP) para monitorización *in vivo***

La presencia de células madre en el tejido medular de individuos adultos (progenitores neurales o epSPCs) abre un abanico de posibilidades en lo que se refiere al tratamiento de lesión medular. No sólo supone un avance en terapia celular sino también en la estimulación del potencial endógeno regenerador del sistema nervioso. Estos últimos años se han llevado a cabo variedad de estudios en los que se pretendía estudiar el comportamiento de estas células madre o progenitores neurales en homeostasis o tras una lesión. En ellos se utilizan técnicas como la farmacología, el silenciamiento con RNAi o sobreexpresión con vectores de expresión en mamíferos para potenciar o deprimir cascadas de señalización celular que aumentan ese potencial endógeno regenerador.

La terapia celular con estos progenitores también es importante ya que ayuda a los mecanismos internos en su función de reparar el daño y proteger las neuronas dañadas tras la lesión medular. Para su estudio es necesario, por tanto, marcar estas células, que van a ser trasplantadas, con una señal fluorescente estable como por ejemplo GFP. De esta manera se podrá hacer un seguimiento de la migración de las células diferenciadas *in vivo*. En primer lugar para observar la migración desde el lugar de inyección hasta el lugar de la lesión, y en segundo lugar para comprobar que se incorporan a la estructura tisular de la misma manera que lo harían de forma natural y no formando estructuras indeseadas.

***Palabras clave:***  lesión medular, terapia celular, progenitores neurales (epSPCs)

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**Generation of a neural stem progenitor cell lines (NPSCs), transfected with Green Fluorescent Protein (GFP) for *in vivo* monitoring**

The presence of stem cells in the adult spinal cord (neural progenitor or epSPCs) entails an important progress in spinal cord injury treatment. This progress is not only related to cell therapy but also to enhance the endogenous regenerative potential of the nervous system. In the last few decades, the attention has been focused on studying the behavior of this stem cells or epSPCs in homeostasis or after the injury. Pharmacological studies, silencing with RNAi and overexpressing analysis with expression vectors in mammals are currently an excellent tool in order to increase or decrease cellular signaling pathways which upgrade this endogenous regeneration potential.

Neural progenitors or epSPCs have an important role on cell therapy. Those stem cells help endogenous mechanism related to damage repairing and neural protection after spinal cord injury. It is, indeed, needed to insert a stable fluorescent marker, GFP for example, to these cells in order to study and monitor *in vivo* differentiated stem cells when transplanted. In first place, to observe the migration from the injection point to the lesion area and, in second place to check if cells incorporate themselves to the tissue structure in a natural way or if they form undesirable structures.

***Key word:***spinal cord injury, cell therapy, neural progenitor (epSPCs)

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# LIST OF ABBREVIATIONS

**SCI**

**CNS**

**GFAP**

**SC**

**OPC**

**hESCs**

**NSPCs**

**ihPSCs**

**OECs**

**MSCs**

**hUCB**

**BMSCs**

**GCV**

**epSPCs**

**CSPG**

**mRNA**

**iPS-NPs**

**EAEC**

**CEBA**

**BOE**

**HSV-TK**

**ULA-6MW**

**pMXIE**

**GFP**

**pHCMV-AmphoEnv**

**HEK 293**

**FBS**

**HEPES**

Spinal Cord Injury

Central Nervous System

Glial fibrillary acidic protein

Stem Cell

Oligodendrocytes Precursor Cells

Human Embryonic Stem Cells

Neural Stem Progenitor Cells

Induced human Pluripotent Stem Cells

Olfactory Ensheatihng Cells

Mesenquimal Stem Cells

Human Umbilical Cord Blood

Bone Marrow Stem Cells

Ganciclovir

Ependymal Stem Progenitor Cells

Chondroitin sulphate proteoglycans

Messenger RNA

Induced Pluripotent Stem-Neural Progenitor Cells

Ethics Committee for Animal Experimentation

Ethical Committee for Animal Welfare

Official Spanish State Gazette

Herpes simplex virus-thymidine kinase

Ultra-Low Attach 6 Multi Well plate

pMX-IRES-EGFP

Green Fluorescent Protein

Amphotropic: capside retrovirus

Human Embryonary Kidney 293

Fetal Bovine Serum

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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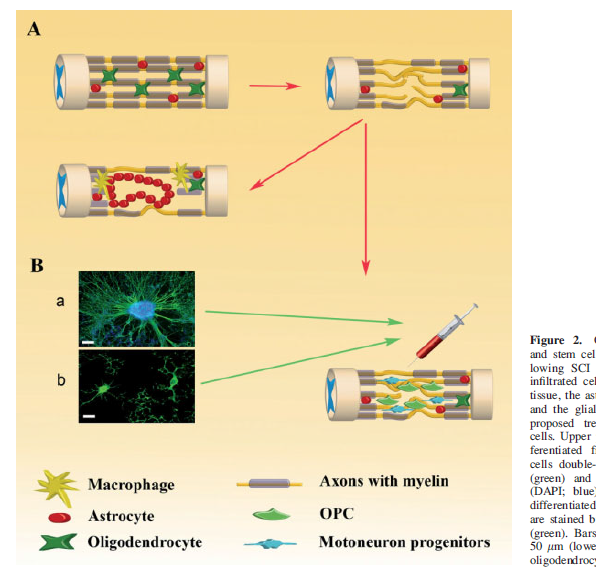
## INTRODUCTION

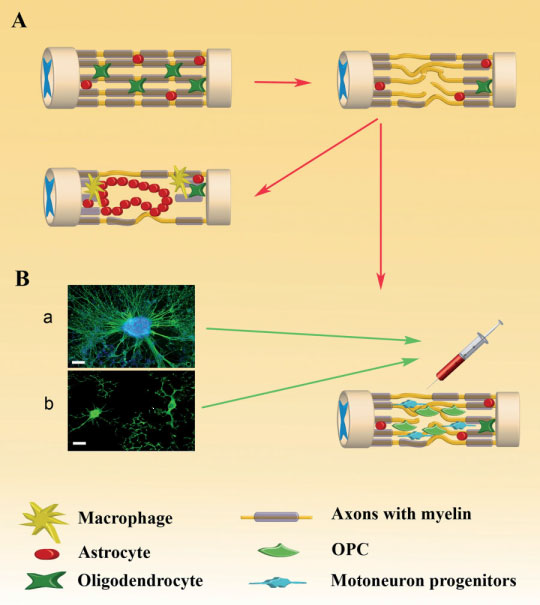
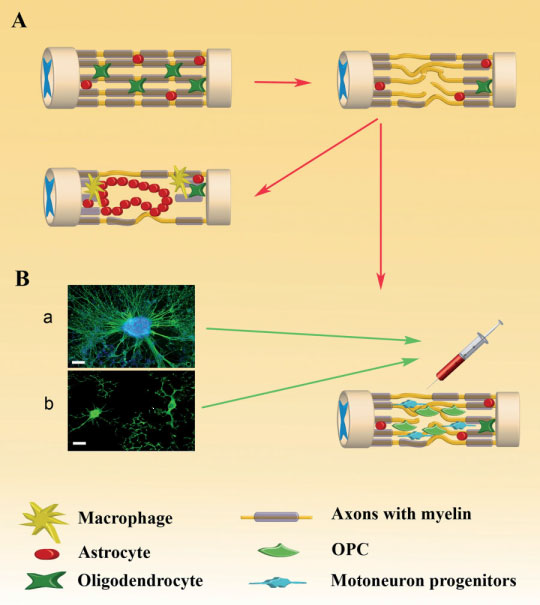
### Stem Cells applied to spinal cord injury

Spinal cord injury (SCI) is a condition which implies devastating physical as well as psychological consequences in the central nervous system (CNS) (Batista et al., 2014). It can be caused by contusion or compression (Rowaland et al., 2008). The cascade of reaction and events taking place after the damage should be introduce for a better understanding of the new approaches using different stem cells. The SCI pathophysiology can be summarized in two complex phases:

* Primary injury phase: appears when both type of lesion occur. After SCI the homeostasis is broken leading to altered ion balance (Young et al., 1986), lipid peroxidation (Braughler et al., 1985) and glutamate release (Rothman et al., 1986). Furthermore, local ischemia contributes to secondary degeneration (Balentin et al., 1978).
* Secondary injury phase: is focused on cellular events such as (a) massive cell death due to the host immune system responses to the injury, (b) secondary necrosis and/or apoptosis, (c) oxidative damages after SCI, (d) excitotoxicity, and (e) axonal damages (Ronaghi et al., 2010). At the end, the progression of the damage contributes to the formation of a scar with fibrotic and a glial compartment made of reactive astrocytes expressing glial fibrillary acidic protein (GFAP; Barnabé-heider et al., 2010; Stenudd et al., 2014).

The purpose of new stem cell (SC) therapies is to avoid the formation of that scar which is at the same time beneficial for re-establishment of physical and chemical integrity of the CNS but implies an important obstacle for neuroregeneration. Figure 1 shows a general approach of what has been doing until now regarding cell therapy (Ronaghi et al., 2010).





**Oligodendrocyte**

**Astrocytes**

**Macrophage**

**Axons with myelin**

**OPC**

**Motoneuron progenitor**

Figure 1. Overview of pathophysiology and stem cell treatment for SCI. (A): Following SCI the BBB is broken and the infiltrates cells from the blood invade the tissue, the astrocytes and glia are activates, and the glial scar and cysts form. (B): A proposed treatment for SCI using stem cells. Upper panel show motoneurons differentiated from human embryonic stem cells double-stained by β-tubulin type III (green) and 4’,6-diamidino-2-phenylindole (DAPI; blue). Lower panel show OPCs differentiated from neuronal stem cells that are stained by oligodendrocyte marker RIP (green). Bars=16µm (upper panel) and 50 µm (lower panel). Abbreviations: OPC, oligodendrocytes precursor cells (from Ronaghi et al., 2010).

The cell loss occurring in SCI points the cell therapy as an effective treatment for axonal regeneration and neural protection (Lukovic et al., 2012). Hence, different SCs have been studied for their application in SCI. Some of them are (Volarevic et al., 2013; Dedeepiya et al., 2014):

* Human Embryonic Stem Cells (hESCs)
* Neural Stem Progenitor Cells (NSPCs)
* Induced human Pluripotent Stem Cells (ihPSCs)
* Olfactory Ensheatihng Cells (OECs)
* Mesenquimal Stem Cells (MSCs)
* Human Umbilical Cord Blood (hUCB)
* Bone Marrow Stem Cells (BMSCs)

However, none of these therapies is completely perfect (Ronaghi et al., 2010; Dedeepiya et al., 2014). Although the drawbacks exist, animal and clinical studies are in progress (Dedeepiya et al., 2014). Advantages and disadvantages related to them are briefly shown in Table 1.

Table 1. Overview of major cell sources employed in the cell-based applications for SCI (Dedeepiya et al., 2014)

|  |  |  |
| --- | --- | --- |
| Type of cell source | Advantages | Disadvantages |
| hESC | Ability to differentiate into various cell linages, ability to proliferate over several passages | Immunorejection (need for immunosuppression) ethical issues, risk of teratoma |
| hUCB | Ease of accessibility | Need for HLA-matched donors, immunosuppression |
| OECs | Ability to support neurogenesis, reduced risk of hypertrophy of the CNS astrocytes, autologous transplantation possible (no graft rejection and no need for immunosuppression), easy accessibility | Differentiation potential is limited when compared with hESC, inadequate cell source particularly in autologous transplants, cell purification difficult |
| BMSCs | Option of using autologous  stem cells and hence lesser  chance of immunorejection | Purification is difficult,  differentiation potential limited |
| NSPCs | Tissue of origin similar and  hence differentiation potential  to neurons is relatively high | Isolation and directed  differentiation difficult |
| ihPSCs | Personalized cell therapy  possible, differentiation potential  similar to ESCs | Immunogenicity, high levels of  genomic instability |

### Mouse as an animal model for SCI

Mouse, *Mus musculus,* is a powerful system for mammalian genetic and biomedical research. (Figure 2). Mice provide effective models due to the physiology shared with humans (Nei et al., 2001; Nguyen and Xu, 2008). It is an ideal model for modeling complex human diseases and drug efficacy testing. Hypertension, diabetes, osteoporosis, glaucoma, neurological and neuromuscular disorders as well as cancer and other rare diseases are some of the conditions for what mice are in used (Paigen and Eppig, 2000).



Figure 2. Mus musculus (extracted from The Jackson Laboratories©)

Some advantages of the mouse as a model organism are (Nguyen and Xu, 2008):

* Genetic manipulation of the mouse genome
* Identification of causative mutations in the mouse genome
* Characterization of genetic background effects
* Value for inbred strains and strain panels

Regarding spinal cord injury, an elegant model was developed by Sofroniew (Faulkner et al., 2004) that allows conditional ablation of GFAP using herpex simplex virus type 1-thymidine kinase (HSV-TK; suicide gene) that is under control of mouse GFAP promoter (Faulkner et al., 2004). This model allows temporary ablation of dividing scar-forming reactive astrocytes using antiviral drug ganciclovir (GCV) in combination with different types of injury (Bush et al. 1999; Faulkner et al., 2004; Voskuhl et al. 2009).

The creation of this model arises from the controversy of the protective role of scar-formation reactive astrocytes (Sofroniew et al., 2005 and 2009; Faulkner et al., 2004), especially in synergism with transplanted cells (Lukovic et al., 2013). This mouse has been already used in SCI model (Faulkner et al., 2004) proving that reactive astrocytes are essential in tissue and neuron protection as well as damage repair. Thus, there are particular interests on the potential of transplanted cells in this model (Erceg et al., 2010).

### Neural Stem Progenitor Cells

Over the past few decades, there were findings of endogenous SCs on specialized niches of the CNS (Agrawal and Schaffer, 2005; Conti and Cattaneo, 2008), presenting morphological and functional heterogeneity in each of the different microenvironments (Qin et al., 2015). These cells are called Neural Stem Progenitor Cells (NSPCs) and have the capacity of giving rise to differentiated neurons (Weiss et al., 1996; Horner et al., 2000; Shihabuddin, 2008). It implies the formation of new circuits contributing to a partial, or even a full, functional recovery after neurological damages (Yamashita et al., 2006). The molecular mechanisms underlying neurogenesis remains elusive but their future discovery will lead to alternative strategies to cell transplantation: *in* situ modulation after injury (Qin et al. 2015).

The endogenous SCs lining the central canal of the spinal cord are the ependymal Stem Progenitor Cells (epSPCs; Meletis et al., 2008; Barnabé-Heider et al., 2010). In homeostasis, its self-renewal capacity is diminished (Johansson et al., 1999). Exclusively, those epSPCs expressing nestin and Sox2 can differentiate into neurons (including GABAergic), astrocytes and oligodendrocytes (Weiss et al., 1996; Gage, 2000; Meletis et al., 2008; Hamilton et al., 2009). Furthermore, under homeostatic conditions, oligodendrocytes maintain self-renewal activity and can give rise to mature oligodendrocytes while astrocytes and epSPCs has limited self-duplication capacity (Johansson et al., 1999; Xu et al., 2006; Meletis et al., 2008). However, after SCI they started to migrate even outside the ependymal layer differentiating themselves into astrocytes and lesser into oligodendrocytes (Figure 3; Meletis et al., 2008; Bernabé-Heider et al., 2010). Both cell types help to protect the neural environment and to restore demyelination, respectively (Okano et al. 2003; reviewed in Ronaghi et al., 2010). Neurons do not proliferate because of the presence of Notch ligand inhibitors (Okano et al., 2003) and Chondroitin sulphate proteoglycans (CSPG; Meletis et al., 2008) from reactive astrocytes (Lukovic et al., 2012)

Distribution uninjured

Generation uninjured (4months)

Net addition injured (4months)



Ependymal cells

Astrocytes

Oligodendrocytes

progenitor

Figure 3. The Relative Contribution of Ependymal Cells, Astrocytes, and Oligodendrocyte Progenitors to New Glial Cells in the Adult. The distribution of ependymal cells (green), astrocytes (red), and oligodendrocyte progenitors (blue) and their progeny are shown at 50% of their estimated number. Note that the right panel depicts the net addition of new cells after injury, and that the addition of the left and right panels gives the full picture of all cells present after the lesion. (extracted from Barnabé-Heider et al., 2010)

Weiss et al. (1996) were the first to describe neurosphere culture and to demonstrate the self-renewal and differentiation capacity of epSPCs *in vitro* (Figure 4). Suspension culture keep intact the self-renewal ability and monolayer culture promotes the differentiation into astrocytes, oligodendrocytes and neurons (Okano et al., 2003; Coutts and Keirstead, 2008). They can be extracted from adult brain, spinal cord and optic nerve of cadavers (Coutts and Keirstead, 2008). In addition, they can be differentiated from ESCs or ihPSCs (Okano et al., 2003; Iwanami et al., 2005; Ronaghi et al., 2010).

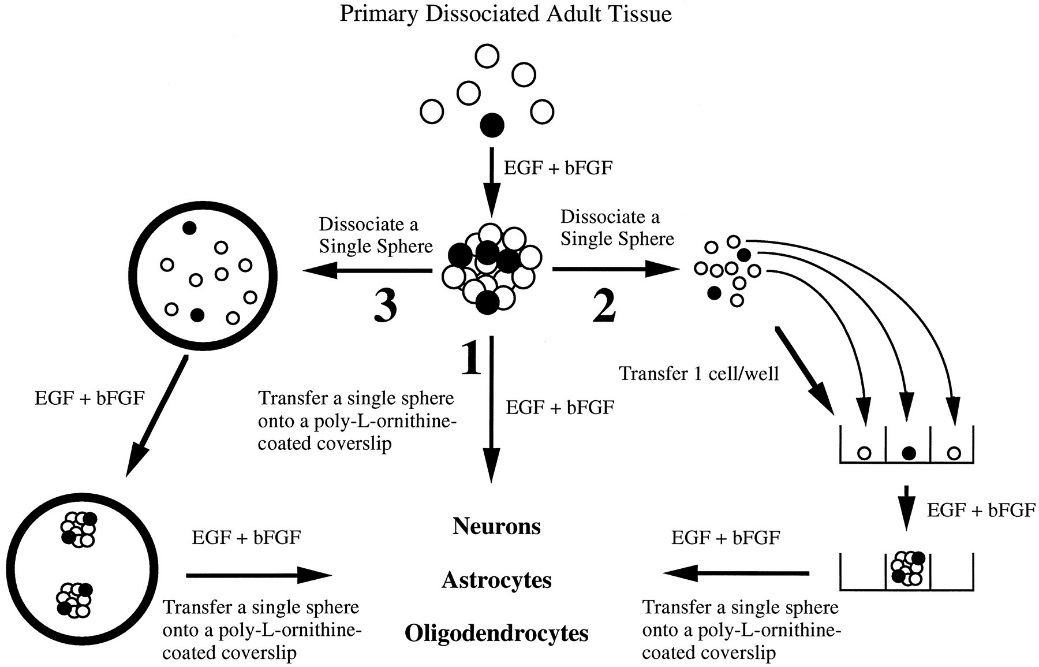


Figure 4. Schematic representation of approaches used to establish adult spinal cord stem cell proliferation, self-renewal and expansion, and production of neurons, astrocytes, and oligodendrocytes. The experimental approaches to demonstrating self-renewal and expansion of stem cells in response to EGF+bFGF are shown. When primary, dissociated adult cells are exposed to EGF+bFGF, spheres of undifferentiated cells are generated. (1) Differentiation of single primary spheres results in the production of neurons, astrocytes, and oligodendrocytes. (2) Dissociation of single primary spheres into single cells, which are plated after serial dilution as 1 cell/well, generates clonally derived secondary spheres. Differentiation of single secondary spheres results in the production of neurons, astrocytes, and oligodendrocytes. (3) Dissociation of single primary spheres into single cells, all of which are plated into one well, results in more than one secondary sphere. Once again, differentiation of these single secondary spheres results in the production of neurons, astrocytes, and oligodendrocytes (extracted from Weiss et al., 1996)

According to previous statements, two different therapeutic approaches have been studyed: recruitment of endogenous NSPCs (Meletis et al., 2008; Moreno-Manzano et al., 2008) or their transplantation (Okano et al. 2003; Ronaghi et al., 2010). In both cases, inhibition of neuronal growth must be taken into account (Okano et al. 2003). Besides, in transplantation, the time at which it is performed plays an important role in functional recovery. Okano et al. (2003), Nakamura and Toyama (2003) and Iwanami et al. (2005) agree in day 9 after injury. In that way, astrocytes and oligodendrocytes have time to form the scar and restore the blood brain barrier (Faulkner et al., 2004; Okada et al., 2006). Table 2 will show a list of pros and cons regarding NSPCs transplants (Coutts and Keirstead, 2008; Ronaghi et al., 2010).

Table 2. Pros and cons of NSPCs transplantation

|  |  |
| --- | --- |
| Pros | Cons |
| Safer than ESCs | Hard to obtain a pure population of differentiated cells |
| No tumorigenic potential | Inefficient tracking system |
|  | Moderate cell survival after transplantation |
|  | Other obstacles regarding axonal regeneration and cell replacement |

Ependymal Stem Progenitor Cells open a new window in SCI treatment (Barnabé-Heider and Frisén, 2008). In comparison with cell therapy, stimulation of endogenous epSPCs is a non-invasive procedure and it avoids immunosuppression (Meletis et al., 2008). However, the natural recruitment of epSPCs after injury does not follow a functional recovery. Therefore, it is still unknown if this kind of therapy could be beneficial or could cause even more damage contributing to the scar formation. Further studies are necessary for a better understanding of their function and their molecular regulation (Barnabé-Heider et al., 2008).

### Induced human Pluripotent Stem Cells

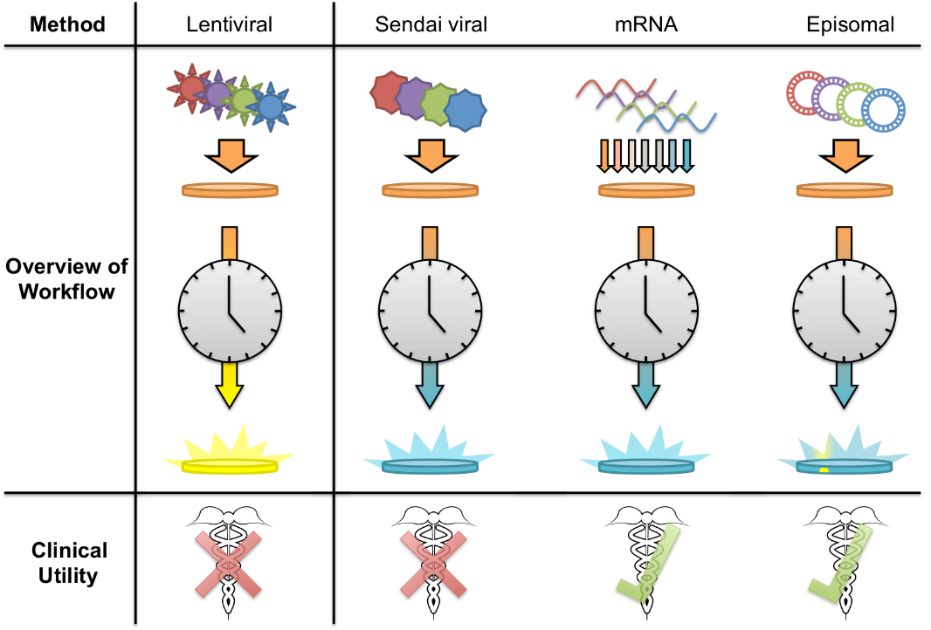
Induced human Pluripotent Stem Cells (ihPSCs) rise as an alternative to hESC. Extracting cells from embryos or fetus leads to ethical issues hard to overcome. Furthermore, high tumorigenic and immunogenic activities are two powerful reasons to find a new approach for cell therapy. The ihPSCs offer the option of autologous transplantation avoiding host rejection (Salewski et al., 2010; Tsuji et al., 2010; Nori et al., 2011; Lucovik et al., 2012).

The ihPSCs come from differentiated tissue reprogrammed by transcription factors so that they create or simulate an embryonic-like state (morphology and differentiation potential). Differences between both cell types, embryonic and induced pluripotent cells, are epigenetic marks (methylation and global expression levels; Salewski et al., 2010; Lucovik et al., 2012) conferring some advantages. The reprogramming issue involves viral vector so that you can introduce those transcription factor genes (Oct4, Sox2, Klf4 and c-Myc; Salewski et al., 2010; Nakamura et al., 2013) in order to confer pluripotency. In other words, resulting cells will exhibit proliferative and differentiation capacity (Nakamura et al., 2013).

The creation of ihPSCs lines increases the risk of tumorigenesis which may be related to foreign provenance of inserted genes or to an incomplete reprogramming (Nakamura et al., 2013). Although the risk exists there are not much studies in which tumor proliferation appears. Using *in vitro* pre-differentiation lines the risk decreases (Romanyuk et al., 2014).

Viral infection and tumorigenesis are the main problems regarding ihPSCs therapy. Hence, several solution have been proposed already applied (Figure 5; Robinton and Daley, 2012; Kramer et al., 2013; Nakamura et al., 2013):

* Transient gene expression (e.g. episomal plasmids, mRNA, Sendai virus…)
* Introducing proteins
* Using drugs instead of genes
* Using minicircle vectors controlling its long-term expression
* Replacing c-Myc and Klf4 oncogenes with NANOG and LIN28 (Yu et al, 2007)



**Method**

**Overview of workflow**

**Clinical utility**

Lentiviral

Sendai viral

mRNA

Episomal

Figure 5. Different protocols to induce pluripotency related to their clinical use (DeVine, 2015)

Regarding the concerns previously mention, each ihPSCs line must be evaluated before clinical application (transplantation). In the case of SCI, ihPSCs will be differentiated into NSPCs (Tsuji et al., 2010; Nori et al., 2011 Lucovik et al., 2012; Nakamura et al., 2013; Romanyuk et al., 2014). Hence, the potential to generate neural cells compared with ESCs must be evaluated.

The evaluation of the efficacy constitutes an important step in ihPSCs transplantation. For SCI treatment, this type of cell line derives into a neural precursor line (iPS-NPs) capable of differentiate into the three main neural linages: astrocytes, neurons and oligodendrocytes (Tsuji et al., 2010; Nori et al., 2011; Lucovik et al., 2012; Nakamura et al., 2013; Romanyuk et al., 2014). In that way, the transplanted cells will be able to migrate, survive and communicate with the host tissue. As a consequence, somatosensory and motor deficits will be reversed. Thus, the evaluation will be focused on axonal regrowth, fulfill lesion cavity and trophic support without tumor formation (Romanyuk et al., 2014).

Romanyuk et al. (2014) as well as Hodgetts et al. (2015) reviewed several cases in which motor and sensorial loss is recovered. However, some other studies failed the purpose of improving this aspect for what Hodgetts et al. (2015) give some reasons: type of injury, rate of maturation of donor cells, inadequate myelination, undesirable ectopic projections and insufficient expression of neurotransmitters.

Summarizing, the next scheme (Figure 6), extracted from Kramer et al. (2013), shows the steps followed in order to use ihPSCs for transplantation.

|  |  |  |
| --- | --- | --- |
| Low reprogramming efficiency |  | High reprogramming efficiency |
|  |  |  |
| Fibroblasts |  | Neural stem cells |

**Cell type**

|  |  |  |
| --- | --- | --- |
| e.g. Oct-4, c-myc\*, klg-4\*, sox-2, Nanog, lin28  (\*increased tumurogenecity) |  | **Alternatives**  Enhancers  Substitutions  Omissions |

**Transcription factors**

|  |  |  |  |
| --- | --- | --- | --- |
| **Genomic Integrations**  **Exision Systems**  **Transient Methods** |  | Risk vs. efficiency vs. labor intensity vs. clinical application |  |

**Mode of Delivery**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Feeder-based** |  | **Feeder-free** |  | **Xeno-free** |
| Efficient platform, proven effective with most cell types, species & reprogramming methods |  | Important for clinical application e.g. mTesR (Stemcell Technologies) Usually requires matrix (e.g. Matrigel or Geltrex) |  | Few studies:   * Stemcell Technologies (mTesR2) * Invitrogen (CellStart) * Millipore (JEScGRO) |

**Culture Platform**

|  |  |  |
| --- | --- | --- |
| Human Cell Lines |  | Non-Human Cell Lines |
| * Morphology * Plutipotent Markers: IHC, PCR ¬gene arrays * Telomerase, Alkaline Phosphatase activity * Teratoma formation |  | * Same as human with addition of: * Chimera formation * Tetraploid complementation |

**Authentication of Pluripotency**

|  |  |  |
| --- | --- | --- |
| Optimal Cell Type? |  | Non-Human Cell Lines |
| Astrocytes  Neurospheres  Oligo Precursor Cells |  | * Differentiation efficiency/propensity * Purity of population – tumor concerns * Markers (GFP, other?) |

**Desired Differentiation Phenotype**

Figure 6. Steps followed in order to use ihPSCs for transplantation (extracted from Kramer et al., 2013)

### Transfection methods

Transfection is a common tool to analyze the characteristics of cloning genes, analyze gene expression, etc. There are numerous techniques in order to introduce a particular gene inside a mammalian cell (Table 3; Washbourne and McAllister, 2002; Kingston, 2003; Karra and Dahm, 2010; Kaestner et al., 2015).

Table 3. Techniques of mammalian cell transfection (Karra and Dahm, 2010; Kaestner et al., 2015)

|  |  |  |
| --- | --- | --- |
| Classification 1 | Classification 2 | Techniques |
| Recombinant virus-based technologies |  | Herpes simplex virus |
| Adenovirus |
| Adeno-associated virus |
| Vaccinia virus |
| Lentivirus |
| Semliki-Forest virus |
| Retrovirus |
| Non-viral transfection methods | Chemical transfection | Calcium phosphate coprecipitation |
| Liposomes |
| Dendrimers |
| Physical transfection | Microinjection |
| Biolistics |
| Electrical Transfection | Electroporation |
| Nucleofection |

Focusing now on viral transfection, it must be said that it is commonly used for *in vivo* assays because its cell specificity capacity (Kingston et al., 2003) but also for *in vitro* experiments like stable genomic integration and inducible expression of transgenes. Choosing a specific virus depends on the gene of interest, the target cell type and the experimental application (Karra and Dahm, 2010). Moreover, five purposes must be accomplished (Washbourne and McAllister, 2002):

* High efficiency in transfecting the desired cell line
* Constructs of varying size, including multiple constructs, should be capable of being transfected
* Limited cellular toxicity
* Easy and safe to perform
* Specifically for SCs, a high titration is required (Pears et al., 1993)

Lentiviral and retroviral vectors belong to the genera of the *retroviridae.* They are among the most powerful techniques for gene delivery into mammalian cells. (Romano et al., 2000; Tonini et al., 2004). Production system of both vectors is fast, reliable and safe (Pear et al, 1993; Soneoka et al., 1995; Romano et al., 2000).

On the one hand, retroviruses have kept the attention of scientific community due to its biological features. Retroviral genome is relatively simple and infect specifically diving cells. As a delivery system they are interesting because they need relatively high titer, have a broad cell tropism, lead to a stable gene expression due to viral genome integration into cell chromosomes, have a no toxic effect on infected cells and the total insert capacity is around 10kb. However, some possible adverse effect could occur as random insertion of viral genome, which may possibly results in mutagenesis and replication competent virus formation by homologous recombination.

On the other hand, lentiviruses can also infect non dividing cells which is a clear advantage for slow or non-replicative cells. The rest of the characteristics are similar to those of retroviral vectors (Romano et al., 2000; Yin et al., 2015).

### Objectives

* Obtaining a clean culture of epSPCs.
* Knowing the transfection capacity of epSPCs when using a retroviral vector.

## MATERIALS AND METHODS

### Animals

Mice were bought to JAX® Mice (The Jackson Laboratories©; Figure 7). They are used in order to extract their spinal cord. Middle age mice (2 months) were selected for this purpose. The Ethics Committee for Animal Experimentation (EAEC) from the Foundation Prince Felipe Research Center approved this study. Moreover, the Ethical Committee for Animal Welfare (CEBA) evaluated the project assigning the number 14016. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013).



Figure 7. Mice placed at their cage with food and water access

In this study, there are two separate mouse strains: a transgenic and a wild type (the control group) (Table 4). The transgenic strain is hemizygous for the insert: HSV-TK. Cells that express HSV-TK will metabolize GCV to toxic nucleotide analogues and undergo cell death. Transgene-derived HSV-TK is present exclusively in cells expressing endogenous GFAP, for example, brain astrocytes or neural stem cells.

Table . Mice strains

|  |  |  |
| --- | --- | --- |
| Mice type | Strain name | Stock number |
| Transgenic | B6.Gg-Tg(Gfap-TK)7.1Mvs/J | 005698 |
| Control | C57BL/6J | 000664 |

### Neurospheres

#### Spinal cord extraction and primary culture

In order to obtain a primary culture of epSPCs in the form of neurospheres we follow the procedure previously described by Reynolds et al. (2005), the first step is extracting the spinal cord from our animal model. The spinal cord must be carefully extracted so that no hair is adhered to it. Hair can cause bacterial or fungal contamination. Furthermore, several cleaning steps must be perform to reduce contamination risk.

Once the spinal cord is extracted, the three layer of meninges are removed to avoid crossed contamination. The next step is the mechanic digestion of the clean spinal cord. The homogenized solution is centrifuged for 5 minutes at 1200rpm and resuspended in new complete medium (Table5 and 6) with mitogen agents. Then it is placed in a 60mm plate for 5-7 days.

Table 5. Complete medium. Supplementation of neurosphere culture medium

|  |  |
| --- | --- |
| Products | Final concentration |
| DMEM/F12 (gibcoTM) |  |
| Hepes 1M (Fisher Scientific) | 5mM |
| NaHCO3 7,5% (Fisher Scientific) | 0,1% |
| Glucose 30% (Fisher Scientific) | 0,6% |
| P/S 100X (gibcoTM) | 1X |
| Fungizone (gibcoTM) | 250µg/mL |
| L-Glutamine 100X | 1X |
| Hormone Mix 100X (Table 6) | 10% |
| Insulin 10mg/µL (Sigma-Aldrich©) | 0,02mg/mL |
| BSA 2g/10mL (BDH Prolabo®) | 4 mg/mL |
| Heparine 375U/mL (Hosporina) | 0,7U/mL |
| EGF (gibcoTM) | 0,0004% |
| FGF (gibcoTM) | 0,0006% |
| Gentamycine (gibcoTM) | 0,001% |

Table . Hormone Mix 100X

|  |  |
| --- | --- |
| Products | Final concentration |
| DMEM/F12 (gibcoTM) |  |
| Hepes 1M (Fisher Scientific) | 4mM |
| NaHCO3 7,5% (Fisher Scientific) | 0,09% |
| Glucosa 30% (Fisher Scientific) | 0,5% |
| Progesterona 2mM (EMD Millipore) | 0,16nM |
| Selenio Sódico 1mM (Fisher Scientific) | 240nM |
| Holo-Transferrina (Sigma-Aldrich©) | 0,8mg/mL |
| Putrescina 0,5M (Sigma-Aldrich ©) | 90µg/mL |

After 5-7 days of the primary culture neurospheres start to form and the complete medium must be changed. Neurospheres are grown in the complete medium so the way to clean the culture is collecting that medium and centrifuging it for 5 minutes at 1200rpm. The resulting pellet is resuspended in complete medium and seeded in an Ultra-Low Attach 6 Multi Well plate (ULA-6MW); 3 wells per spinal cord. The same procedure is performed each 2 or 3 days until the culture is completely clean or you want to start the infection experiment.

### Infection

In order to perform the infection of epSPCs three main aspect are described: plasmid construction, plasmid purification and infection.

#### Plasmids

* pMX-IRES-EGFP (pMXIE)

IRES

GFP

3’ LTR

5’ LTR

ψ

AmpR

pUC ori

MCS

pMXIE

7.2 kb

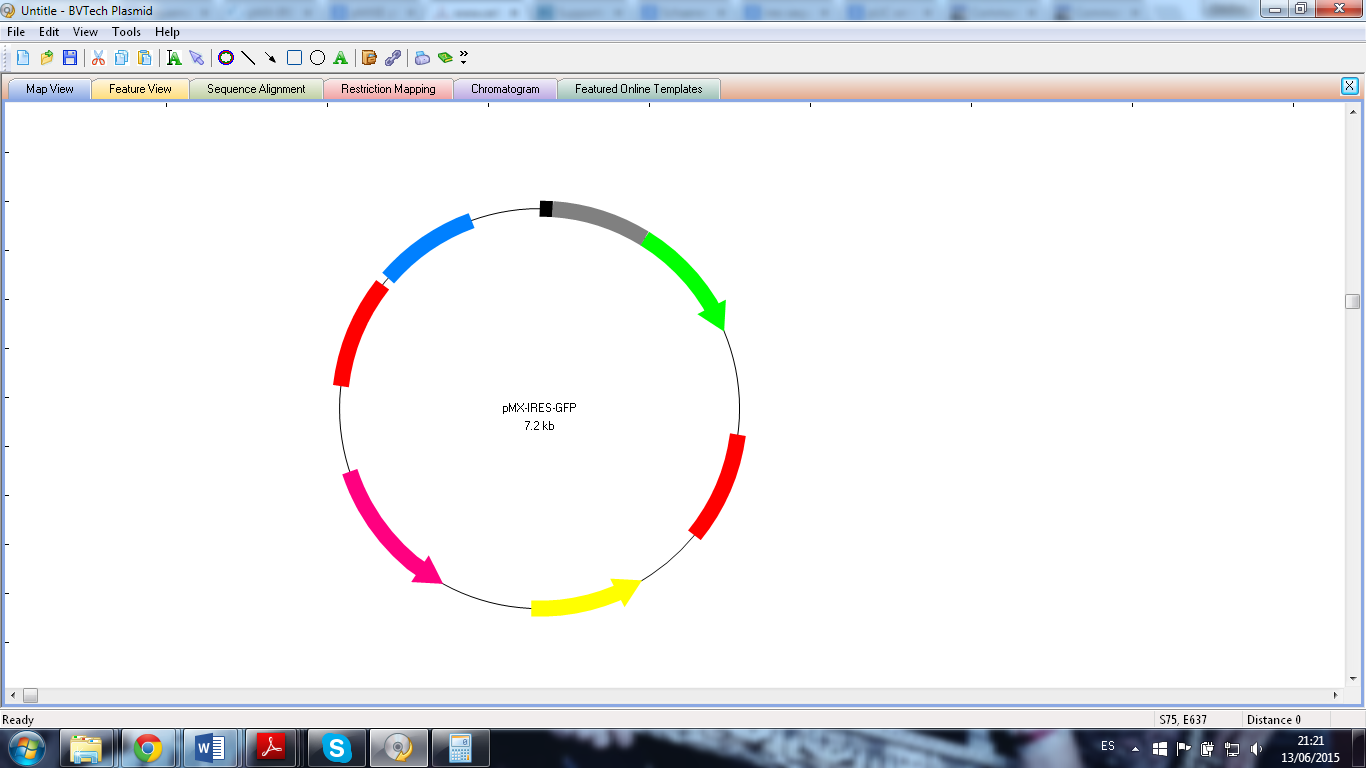
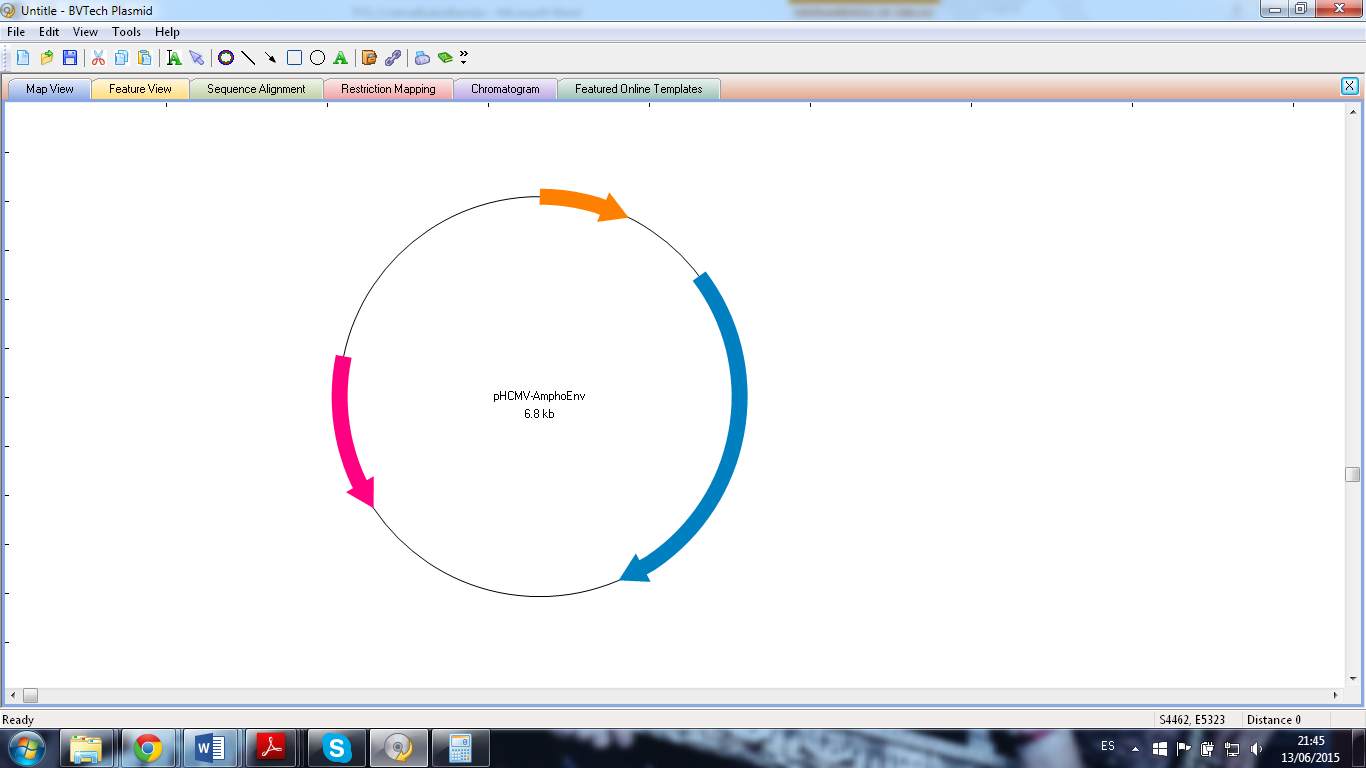


Figure 8. pMXIE. Plasmid containing the GFP gen.

* Amphotropic: capside retrovirus. pHCMV-AmphoEnv

pHCMV-AmphoEnv

6804 bp

AmpR

MLV Amphotropic

CMV

Figure 9. pHCMV-AmphoEnv. Plasmid containing retrovirus envelope or capside.

#### Plasmid purification

Two different cultures are performed in order to grow bacteria containing plasmids. They are purified using the Genopure Plasmid Maxi kit® (Roche©), high-copy number plasmid protocol. Briefly, bacterial culture is centrifuged and cells are lysated. The suspension is cleared by filtration or centrifugation. The plasmid is purified thanks to a column through which the clear solution is passed. Then, DNA is isolated by isopropanol and ethanol steps.

#### Transfection of HEK 293 cell line, virus collection and infection of epSPCs

The method chosen to transfect HEK 293 cell line with both plasmids was a chemical transfection with liposomes. The resulted cells become a virus factory. The second transfection, infection, will be done to epSPCs with the retroviruses previously collected.

Firstly, a culture of 3x106 HEK 293 cells is seeded in a 100mm plate. The next day, 20 minutes before transfection, this culture must be exposed to a free-antibiotics complete medium (DMEM High glucose (gibcoTM), 3% FBS (biowest) and 1% Penicillin/Streptomycin (gibcoTM)). The transfection solution contains OPTIMEM®, lipofectamine 2000 (InvitrogenTM) and both plasmid in a 1:1 proportion. The mixture is incubated at room temperature for 20 minutes and then is added to the plate drop-wise. After 24 hours, the medium is replaced by the complete medium of the cells you want to transfect. Next day, the supernatant of HEK 293 is collected and replaced by fresh medium. To collect the viruses the supernatant is centrifuged for 5 minutes at 3000rpm. Again, the supernatant must be took out carefully in order to not disrupting the pellet of HEK 293 cells. A mixture of the supernatant, HEPES 1M and polybrene solution 1mg/mL must be done and added to epSPCs. Infection is repeated three times in the next two days.

The same procedure is done with a second virus factory, starting one day later.

### Fluorescence assay

Viral transfection efficiency is evaluated under the fluorescence microscope (blue filter, Ph1, 5X). The number of total neurospheres and the green ones are counted. Then, the percentage of transfected neurosphere is estimated.

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## RESULTS

The first objective has been overcome. Clean neurospheres were perfectly isolated from mouse spinal cord after three months of growing the baby mice, extract the spinal cord and pass the culture. Figure 10 shows the aspect of the clean culture of neurospheres.

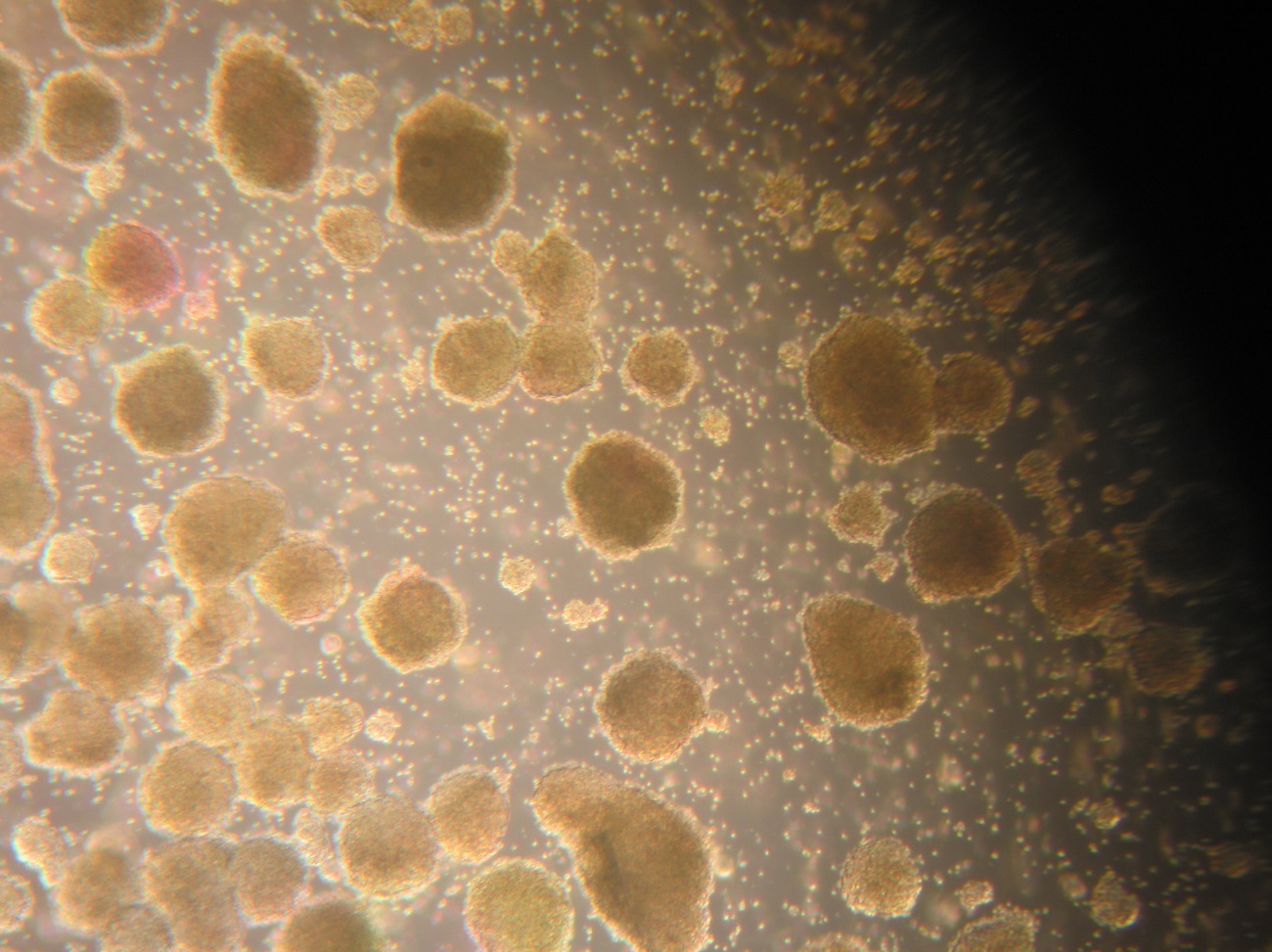


Figure 10. Clean culture of neurosphere (5X)

The viral transfection of epSPCs was performed with a total of four individuals. One of them was wild type and the other three were transgenic (HSV-TK). There is no difference between both types until ganciclovir is not administered. So we consider all experiments will behave the same way. The efficiency reach regarding the four experiment already mentioned has an average value of 41.9% ± 7.9% (Table 7).

Table 7. Efficiency of viral transfection of epSPCs

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Experiment/Counting | 1 | 2 | 3 | 4 |
| Total | 405 | 311 | 101 | 130 |
| Infected | 183 | 160 | 33 | 53 |
| Percentage | 45,2% | 51,4% | 32,7% | 40,8% |
| Media |  |  |  | 41,9% |
| Standard deviation |  |  |  | 7,9% |

Figures 11, 12 and 13 show some of the neurospheres, either wild type or transgenic, transfected by the retrovirus.

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Figure 11. Infected neurosphere with GFP via retroviral vector (1). Among all neurosphere in the captured area those ones presenting green point of fluorescence have been successfully infected. Picture was taken at zoom 5X.

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Figure 12. Infected neurosphere with GFP via retroviral vector (2). Among all neurosphere in the captured area those ones presenting green point of fluorescence have been successfully infected. Picture was taken at zoom 5X.

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Figure 13. Infected neurosphere with GFP via retroviral vector (3). Here we can see how this two big neurosphere are greatly infected. Picture was taken at zoom 5X.

## DISCUSSION

Gene transfer via viral infection is a powerful tool for long lasting expression (Kingston, 2003; Karra and Dahm, 2010). In particular, it is used in neurosphere in order to elucidate molecular pathways related to regeneration, study gene expression or evaluate the behavior under certain circumstances (Bartrem et al., 2010; Serrano-Pérez et al., 2015). In this particular case, we are inserting a sequence which encodes the GFP because the aim of the project is creating a cell line of green NSPCs usable for monitoring *in vivo* the migration, differentiation and integration of those cells. For that reasons, pluripotency and differentiation capacities should be maintained after infection as it is proved by Bartrem et al. (2010). This line will be a potent tool for further studies of CNS regeneration.

Different viral vector have been used for gene delivery into neurospheres, some examples are adenovirus (Fu et al., 2008; Bartrem et al., 2010), lentivirus (Suzuki et al., 2002; Zhou et al., 2015) or retrovirus (Yamamoto et al., 2001). Hence, different efficiencies were achieved. Bartrem et al (2015) conclude that for a good infection the viral titration must not interfere neither in cell viability nor neurosphere reconstitution. Then, correct efficiency was a 50%. Furthermore, they also postulate that dissociation of neurospheres increase the efficiency so the viral titration may be reduced. Suzuki et al. (2002) and Zhou et al. (2015) reach around an 80% of efficiency with a lentivirus while Yamamoto et al. (2001) poorly get a 10% of infection employing a retrovirus. In comparison, we reach a 41.9% of infection, much higher than the obtained in Yamamoto et al. (2001) experiments. However, lentiviral transfection probably could achieved a higher efficiency because they not only infect dividing cells but also non-dividing ones (Romano et al., 2000; Yin et al., 2015).

As previously mention, neurosphere dissociation increases efficiency of infection so it is reasonable to think that the efficiency was dependent on the size of the neurospheres, in other words, virus penetration capacity is limited (Bartrem et al., 2010). Besides, this might be the explanation to the fact that the majority of neurospheres are not completely green but they only present green point inside them.

## CONCLUSION

Neurospheres was successfully isolated from our animal model, allowing their transfection.

Employing this methodology, around a 40% of epSPCs are infected with GFP. It provides a potent tool for further studies in differentiation, migration and integration of epSPCs directly related with regeneration and replacement therapies.

## FUTURE PERSPECTIVES

After transfecting epSPCs with GFP there are some other issues that must be done to continue the project. On the one hand, neurosphere would be used for cellular transplantation in order to see the migration of differentiated cells *in vivo*. For that purpose green and non-green cell populations are separated by sorting.

On the other hand, ihPSCs would be transfected to repeat the process we followed using epSPCs: *in vivo* monitoring of those cells. GFP transfection must be fine tunning because nobody else has transfected them before with a viral vector. The way researchers have accomplished this goal was by inserting GFP sequence in the same construction of pluripotency transcription factors when reprogramming somatic cells (Stadtfeld et al., 2008; Kim et al., 2012) or by nucleation and liposome as Chartterjee et al. (2011) did.

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