Universidade de Aveiro Secção Autónoma de Ciências da Saúde
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Sofia Duarte Boia de Oliveira Carvalho

Modelos baseados em células estaminais para estudo de neuropatologias

Establishing stem cell based systems to study neuropathologies

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Odete Abreu Beirão da Cruz e Silva, Professora Auxiliar com Agregação da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro e sob a co-orientação da Professora Doutora Sandra Maria Tavares da Costa Rebelo, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, irmão e ao André.

o júri

presidente

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palavras-chave

Células estaminais, Mucosa olfactiva, Neuroesferas, Células olfactivas derivadas de neuroesferas, Células tipo neuronal

resumo

As células estaminais distinguem-se dos outros tipos de células pela sua capacidade de auto-regeneraração e simultaneamente diferenciação em diferentes tipos celulares. Estas células dividem-se em duas categorias: células estaminais embrionárias e somáticas. As que se enquadram no primeiro grupo originam todos os tipos celulares de um determinado organismo (pluripotentes), enquanto as células estaminais somáticas originam apenas alguns tipos celulares (multipotentes). Atualmente, células diferenciadas podem no entanto ser geneticamente reprogramadas para um estado indiferenciado através da indução de expressão de genes específicos que estão altamente expressos em células estaminais embrionárias (células estaminais de pluripotência induzida).

A possibilidade de estudar neuropatologias utilizando modelos baseados em células estaminais tem sido amplamente explorada nos últimos anos. Como tal, os principais objetivos desta dissertação foram o isolamento e proliferação de células estaminais da mucosa olfativa e a sua posterior diferenciação em células derivadas de neuroesferas olfativas (ONS) e células tipo neuronal (NLC). A caracterização do sistema modelo ONS foi igualmente realizada. Sequencialmente, foram utilizadas duas enzimas (Dispase e Colagenase) para isolar as células estaminais da mucosa olfativa. Células estaminais da lâmina própria e do epitélio da mucosa olfativa foram isoladas e proliferaram no meio DMEM/F12. Ambos os tecidos foram diferenciados em neuroesferas (utilizando meio DMEM/F12 suplementado com ITS-X, EGF e FGF2) e posteriormente em células derivadas de neuroesferas olfativas (utilizando meio DMEM/F12 suplementado com FBS) e em células tipo neuronal (usando meio neurobasal suplementado com B27, glutamina e glutamato). Os nossos resultados indicam que estabelecemos com sucesso culturas primárias de células estaminais olfativas a partir da mucosa olfativa de rato. A eficiência dos processos de isolamento e proliferação foi comprovada pela presença do marcador de estaminalidade nestina. A diferenciação das células estaminais em células derivadas de neuroesferas olfactivas (ONS) também for realizada com sucesso. A caracterização bioquímica dessas células revelou que, relativamente aos níveis de expressão da proteína precursora de amiloide de Alzheimer (PPA) e da proteína Tau, o sistema modelo em estudo apresenta resultados semelhantes aos obtidos com alguns sistemas modelo do tipo neuronal previamente caracterizados, nomeadamente as linhas celulares PC12 e SH-SY5Y. No entanto uma caracterização mais pormenorizada deve ser realizada.

Os resultados obtidos fortalecem a hipótese de este modelo poder vir a ser utilizado para estudo dos mecanismos moleculares subjacentes a diversas neuropatologias, como a doença de Alzheimer.

keywords

Stem cells, Olfactory mucosa, Neurospheres, Olfactory Neurospheres-Derived Cell, Neuron Like Cells

abstract

Stem cells are distinguished from other cell types by their ability to both self-renew and to differentiate into a diverse array of specialized cell types. Naturally occurring stem cells are divided into two categories: embryonic stem cells and somatic stem cells. While embryonic stem cells are able to generate all the differentiated cells of the developing soma (pluripotent stem cells), somatic stem cells assume increasing degrees of fate restriction as they specialize into specific tissue lineages (multipotent stem cells). Specialized cells can also be genetically reprogrammed to a stem cell-like state through the induced expression of key genes that are highly expressed in embryonic stem cells.

The possibility to investigate neuropathologies using stem cells based systems has been widely explored in the last years. Therefore the main objectives of this dissertation were isolation and proliferation of olfactory mucosa stem cells that were further differentiated in olfactory neurospheres derived cells (ONS) and neuron-like cells (NLC). Characterization of the ONS model system was also performed. We sequentially used Dispase and Collagenase to isolate olfactory mucosa stem cells that further proliferate in DMEM/F12 medium. The stem cells of either lamina propria and epithelium of olfactory mucosa were isolated and proliferated. Both tissues were further differentiated in neurospheres (using DMEM/F12 supplemented with ITS-X, EGF and FGF2), and finally in olfactory neurospheres-derived cells (using DMEM/F12 medium) and neuron-like cells (using neurobasal medium supplemented with B27, glutamine and glutamate). Our results indicate that we successfully established primary cultures of olfactory stem cells from rat olfactory mucosa. The efficiency of the isolation/proliferation procedure was accomplished by positive immunostaining using the stemness marker nestin. The differentiation of the olfactory stem cells into olfactory neurospheres derived cells (ONS) was also effective. The preliminary morphological and biochemical characterization of the ONS models system was achieved and revealed that our ONS model system in term of APP and Tau expression levels behaves similarly to neuronal-like model systems previously characterized including PC12 and SH-SY5Y cell lines. However, additional characterization should also be performed. Our results strength the hypothesis of using stem cells based model systems to study the cellular and molecular mechanisms underlying several neuropathologies, including Alzheimer's disease.

ABBREVIATIONS

AOB	Accessory olfactory bulb
AON	Anterior olfactory nucleus
APP	Amyloid Precursor Protein
BC	Basal cells
BCA	Bicinchonic acid
BM-MSCs	Bone marrow mesenchymal stem cells
BSA	Bovine serum albumin
CSCs	Cancer stem cell
CX	Cortex
DMEM	Dulbecco's Modified Eagle Medium
EC	Entorhinal cortex
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGCs	Primordial germ cells
EGF	Epidermal growth factor
EPI	Epithelium
epi SCs	Epiblast stem cells
ER	Enzymatic reaction
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGF2	Basic fibroblast growth factor
GBCs	Globose basal cells
GFAP	Glial fibrillary acid protein
H	Hipothalamus
HBCs	Horizontal basal cells
HLA	Human Leukocyte antigen
HSCs	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
ITS-X	Insulin Tranferrin Selenium Ethanolamine Solution
LA	Lateral amygdala
LB	Loading buffer
LP	Lamina propria
LTP	Long term potentiation

MAB	Monoclonal antibody
MAP2	Microtubule-associated protein
MOE	Main olfactory epithelium
MSCs	Mesenchymal stem cells
NB	Neurobasal medium
NLCs	Neuron like cells
NS	Neuroespheres
NSCs	Neural stem cells
OB	Olfactory bulb
OD	Optical density
OECs	Olfactory ensheating cells
OE-MSCs	Olfactory ecto-mesenchymal stem cells
OM	Olfactory mucosa
ONS	Olfactory neurospheres-derived cells
ORNs	Olfactory receptor neurons
OSCs	Olfactory stem cells
OT	Olfactory tubercle
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered salin
PC	Progenitor cell
PD	Parkinson's Disease
Pen	Penicillin
PN	Passage number
RMS	Rostral migratory stream
ROS	Reactive oxigen species
RT	Room temperature
SDS	Sodium dodecy sulfate
SGZ	Subgranular zone
ST	Stem cell
Strep	Streptomycin
SUS	Sustentacular supporting cells
SVZ	Subventricular zone
SZ	Schizophrenia
TBS	Tris buffered saline
TBS-T	Tris buffered saline - tween
TDC	Terminally differentiated cell
VA	vomeronasal organ
VNO	vomeronasal organ
WR	Working reagent

INDEX

1. INTRODUCTION

1.1 STEM CELLS

The human body comprises over two hundred different cell types that are organized into tissues and organs to provide all the functions required for viability and reproduction (Watt and Driskell, 2010). It is generally the case that cellular potency is progressively restricted as development proceeds from a fertilized egg to the adult. Nevertheless, stem cells are an exception to this rule in that they retain, to varying extents, the potential for multi-lineage differentiation (Daley, 2010). Additionally, one of the most significant properties of this distinct class of cells is their ability to self-renew (Preston et al., 2003). To achieve these two remarkable tasks, the cells can undergo an asymmetric cell division whereby they generate both self-renewing and differentiating daughter cells known as progenitor cells (PC; Fig.1) (Neumuller and Knoblich, 2009). A progenitor, also named precursor cell, represents an intermediary stage during the differentiation process which is capable of undergoing cell division and further specialization in order to generate terminally differentiated cells (TDC; Fig.1).

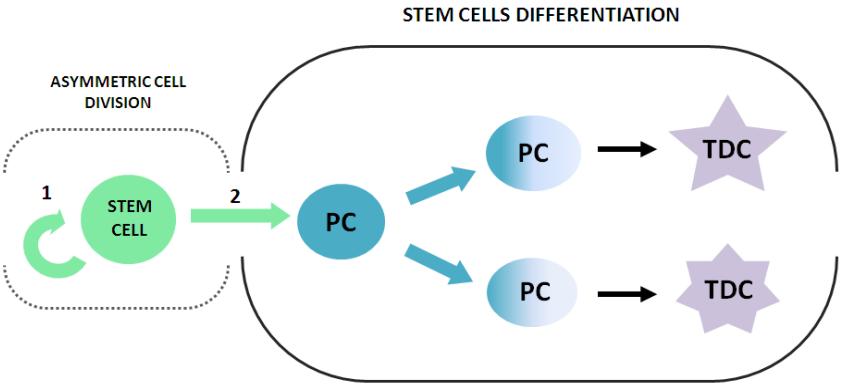

Figure 1 – Differentiation of stem cells into specialized cells. Stem cells can divide asymmetrically to generate both (1) self-renewing and (2) progenitor cells (PC). During stem cells differentiation process, progenitor cells can undergo cell division and further specialization to become a terminally differentiated cell (TDC).

Independently of stem cells type, both self-renewal and the potential for multi-lineage differentiation are strongly affected by their local micro-environment, known as stem cell niche. The niche is generally composed of stem cells and supporting cells (neighboring cells) which are responsible for maintaining homeostasis within the stem population. There are some aspects of the stem cell niche that are known to influence self-renewal and stem cell fate are adhesion to extracellular matrix proteins, direct contact with supporting cells, exposure to secreted factors (such as growth factors) (Watt and Driskell, 2010) and reactive oxygen species (ROS) in stem cells (Li and Bhatia, 2011) (Fig.2).

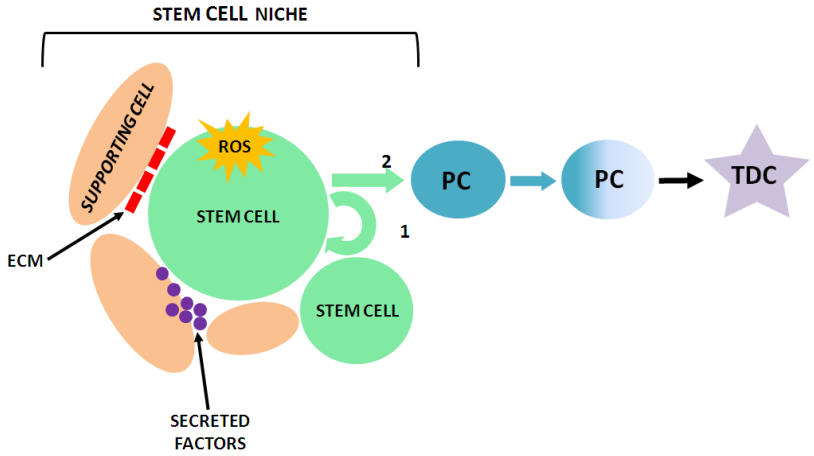

Figure 2 – The stem cell niche. Stem cells are maintained in a specialized environment that is characterized by the presence of supporting cells. Stem cells can undergoing asymmetric division to produce (1) a stem cell that remain in contact with the support cells and (2) a progenitor cell (PC) that does not receive cellular signals from the supporting cells and thus begin their differentiation process to produce terminally differentiated cells. Different components of the stem cell niche are illustrated: supporting cells, extracellular matrix (ECM), secreted factors and reactive oxygen species (ROS).

Another critical concept for the understanding of the function and potential clinical applications of stem cells is the developmental potency. This ranges from the totipotency (ability to form the embryo and the trophoblast of the placenta) of the fertilised oocyte, to the pluripotency (ability to differentiate into almost all cells that arise from the three germ layers) of embryonic stem cells, to the multipotentiality (ability of producing a limited range of differentiated cell lineages appropriate to their location) of somatic stem cells (Daley, 2010).

1.1.1 Embryonic stem cells

In 1981, it was discovered that embryonic stem cells could be derived from the inner cell mass of the mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981) (Fig. 3). In 1998, it was first reported that these cells could be derived from human blastocysts (Thomson et al., 1998) and be differentiated into all cells types composing human body (Kim and Jin, 2012) trough sequential differentiation of the ectoderm, endoderm, and mesoderm germ layers (Verma and Verma, 2011).

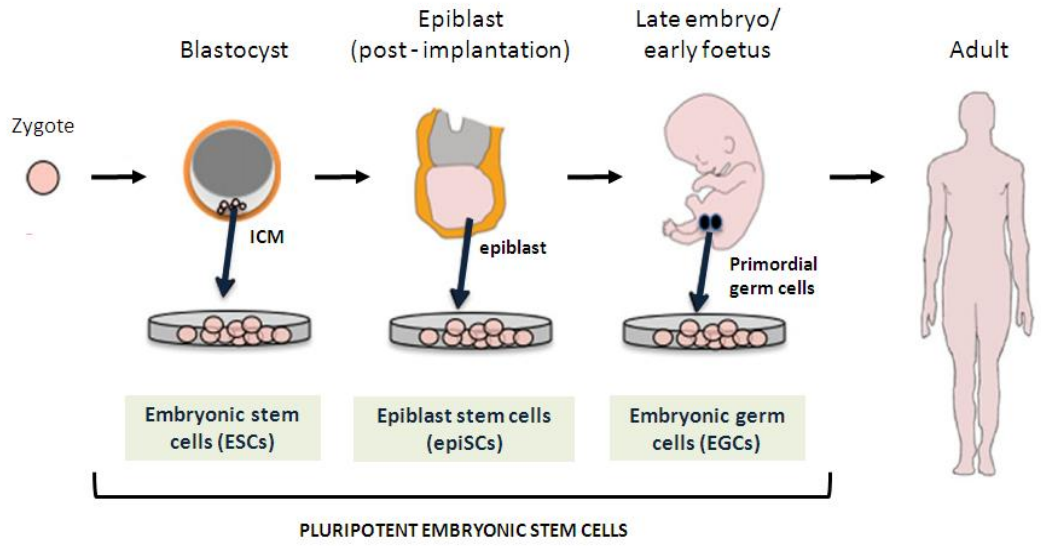

Figure 3 – Origin of the pluripotent embryonic stem cells. ECS cells could be derived from the inner cell mass (ICM) of the blastocysts, from the post-implantation epiblast and from primordial germ cells. ESCs, Embryonic stem cells; epiSCs, Epiblast stem cells; EGCs, Embryonic germ cells Adapted from Watt and Driskell, 2010.

However, blastocysts are not the only source of pluripotent embryonic stem cells. Epiblast stem cells (epiSCs) can be derived from the post-implantation epiblast of mouse embryos (Brons et al., 2007; Tesar et al., 2007). Moreover, pluripotent embryonic stem cells can also be derived from primordial germ cells (EGCs), progenitors of adult gametes, which diverge from the somatic lineage at late embryonic to early fetal development (Kerr et al., 2006).

So far, embryonic stem cell lines have been generated from other species beyond human and mouse; such as chicken, hamster, rabbit, rat and monkey (Wobus and Boheler, 2005).

In January of 2009, just over 10 years after they were first isolated, the US Food and Drug Administration approved the first clinical trial involving human embryonic stem cells in which the safety of ES cell-derived oligodendrocytes in repair of spinal cord injury will be evaluated (Daley, 2010). Nevertheless, the use of embryonic stem cells create legal and ethical problems of destroying embryos (derived from the discarded fertilized ovum during *in vitro* fertilization) and problems after transplantation sine cells derived from ESCs may be rejected from the recipient patients and immunosuppressants are required to be administered after transplantation.

1.1.2 Somatic stem cells

Somatic stem cells (also known as adult stem cells, which invite confusion because they can be extracted from newborns and adolescents and therefore not strictly adult sources) are similar to embryonic stem cells in their ability to terminally differentiate and to self-renew; however, unlike embryonic stem cells, somatic stem cells have a multiple tissue-restricted developmental potency. Nevertheless, somatic stem cells are emerging as particularly strong candidates for cellular therapies; since they overcome barriers concerning to the controversial embryo manipulation as well as the tumorigenicity of embryonic stem cells (Daley, 2010).

Somatic stem cells are tissue-resident undifferentiated cells located in a stable micro-environmental niche which is responsible for maintaining a balance of stem cell

quiescence and activity (Rakesh Sharma Ka, 2009). The quiescent state appears to be necessary for preserving self-renewal capacity of somatic stem cells. Therefore, somatic stem cells are maintained in a quiescent state (out of cell cycle and in a lower metabolic state) but are able to exit quiescence and rapidly expand and differentiate. This remarkable feature is crucial for physiological tissue renewal and regeneration after injury (Li and Bhatia, 2011).

A clear understanding of the intrinsic and extrinsic regulatory mechanisms that control somatic stem cells quiescence and activity is crucial to optimize delivery of stem cell therapies. One area that the study of somatic stem cell quiescence is highly relevant is the cancer therapy. There is mounting evidence that a subset of cells, termed cancer stem cells (CSCs), are responsible for long-term maintenance of tumor growth in several cancers (Dalerba et al., 2007), such as acute leukemia (Wang and Dick, 2005) and solid tumors, including brain, breast and colon tumors (Ailles and Weissman, 2007)). Once quiescent CSCs are often resistant to both conventional chemotherapy and targeted therapies (Li and Bhatia, 2011), it becomes clear that improved understanding of mechanisms of stem cell quiescence is important not only for directed manipulation of normal stem cells function, but also for development of approaches to therapeutically target quiescent CSCs.

According to their own features, somatic stem cells have been divided into different subgroups. From these, the cell types that are well studied and characterized to improve stem cells based therapies are: hematopoietic, mesenchymal, epithelial and neural stem cells.

1.1.2.1 Hematopoietic stem cells

The Hematopoietic stem cells (HSCs) are the archetypal stem cell, from which much of knowledge on stem cells has arisen (Preston et al., 2003). These cells, which were primarily found in the bone marrow, are also present in other tissues including umbilical cord blood and peripheral blood. HSCs are responsible to generate all of the mature blood cells in the body (red blood cells, platelets, and a variety of lymphoid and myeloid cells) and surprisingly, several reports in the last few years suggest that these type of stem cells are able to differentiate into nonhematopoietic cells including hepatocytes, muscle cells, epidermal cells, islet cells, neurons, myocardium, and other lineages under the right environmental conditions (Smith, 2003).

In 1968, the first major landmark in stem cells transplantation from bone marrow occurred with successful allogeneic transplantations. They are performed in infant with X-linked lymphopenic immune deficiency and for another with Wiskott-Aldrich syndrome (Gatti et al., 1968; Bortin, 1970). Lately, due to advances in histocompatability testing and development of marrow donor registries, there is a growing number of patients who can receive transplants (Koo and Ahn, 2012) for treatment of high-risk or recurrent hematologic malignancies, bone marrow failure syndromes, selected hereditary immunodeficiency states, and metabolic disorders (Wagner and Gluckman, 2010). However, only approximately 30% of the patients can receive transplantation from a human leukocyte antigen (HLA)-matched sibling donor. In the absence of such a donor, the search for an unrelated volunteer adult donor is currently performed and the HLA-matched unrelated donors are found for only approximately 50% of the patients. Additionally, the search for donors can take weeks or months (Koo and Ahn, 2012).

In the absence of better treatment strategy, it was considered a new source of hematopoietic stem cells namely, umbilical cord blood (Wagner and Gluckman, 2010). The first human cord blood transplant was performed in 1988 and therefore cord blood banks have been established worldwide for the collection and cryopreservation of umbilical cord blood for allogeneic hematopoietic stem cell transplantation.

The main practical advantages of using cord blood as an alternative source of stem cells are the absence of risk for mothers and donors, reduced likelihood of transmitting infections (particularly cytomegalovirus) and the ability to store fully tested transplants. The human leukocyte antigen (HLA) typed transplants are frozen and are available for immediate use (Gluckman et al., 2011).

Another type of hematopoietic stem cells transplantation is performed from peripheral blood. Intense research have revealed that the low number of circulating stem cells (comprising only 1/10000 to 1/100000 of total blood cells) (Preston et al., 2003) can be increased by injecting the donor with a cytokine such as granulocyte-colony stimulating factor (G-CSF), which forces the stem cells to migrate from bone marrow to peripheral blood (Korbling and Freireich, 2011).

1.1.2.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are nonhematopoietic stromal cells that possess the capacity of self renew and multilineage differentiation, thereby contributing to the regeneration of mesenchymal tissues during lifespan (Chamberlain et al., 2007).

For several reasons, MSCs are emerging as particularly strong candidates for cellular therapies. Firstly, they can be isolated from a wide range of autologous sources (bone marrow (Gnecchi and Melo, 2009; Hao et al., 2010), adipose tissue (Gronthos et al., 2001), peripheral blood (Kuznetsov et al., 2001; Miura et al., 2003), umbilical cord blood (Rosada et al., 2003), tendon (Salingcarnboriboon et al., 2003), ligaments (Seo et al., 2004), olfactory mucosa (Tome et al., 2009; Delorme et al., 2010) and deciduous teeth (Miura et al., 2003); some of which are readily accessible, using robust, well-established techniques (Pittenger et al., 1999; Gronthos et al., 2001; da Silva Meirelles et al., 2006).

Secondly, their high proliferative potential allows rapid MSCs expansion *ex vivo*, while maintaining multipotentiality. Finally these cells are potentially suitable for use in allogeneic as well as autologous transplantation (Aggarwal and Pittenger, 2005).

For all the reasons presented MSCs have deserved further attention as potential candidates for the reparative/regenerative treatment of diseases affecting mesodermal tissues (e.g. bone, cartilage, muscle and kidneys). Additionally, in recent years it become evident that MSCs could be useful for neurological treatments, based on evidence that their transplantation results in functional recovery in various animal models of neurological disorders; even if the mechanism responsible remains unclear (Maltman et al., 2011).

1.1.2.3 Epithelial stem cells

Epithelia are continuous sheets of tightly linked cells that constitute the surfaces (e.g. epidermis and corneal epithelium) and linings (e.g. digestive, respiratory, and uro-genital epithelia) of the body; providing a protective envelope against the external environment and also regulating water and nutrient absorption as well as glandular secretions (Blanpain et al., 2007). Epithelial stem cells located in different adult tissues are essential to sustain tissue turnover and repairment of epithelia upon injuries. Therefore, different populations of epithelial stem cells can generate tissues (e.g. intestine, epidermis, mammary gland and cornea) that display several cellular architectures and distinct functions. The epithelial stem cells niches that have been better characterized are: intestinal crypt, corneal limbus, hair-follicle bulge and mammary gland terminal end bud.

Cases of eye damage have been successfully treated by first culturing limbal stem cells and then transplanting the autologous corneal epithelial sheets (Wylegala et al., 2008; Ahmad et al., 2010)

1.1.2.4 Neural stem cells

It is now widely accepted that in mammals, including humans, newly born neurons are continuously generated from neural stem/progenitor cells, and then incorporated into the functional network of the adult brain. In the adult brain, neural stem cells (NSCs) exist principally in two regions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Imayoshi et al., 2011).

In the SVZ of the lateral ventricle, a large number of neurons generated from stem cells, migrate through the rostral migratory stream (RMS) into the olfactory bulb to differentiate into local interneurons (Fig. 4).

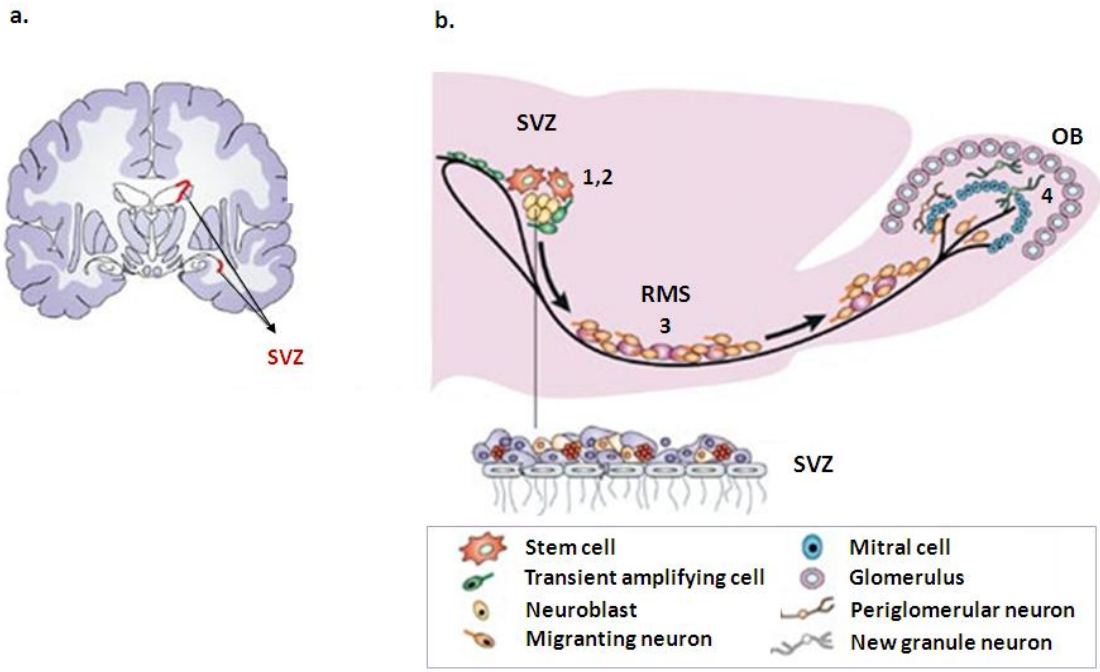

Figure 4 – Generation of new interneurons in the olfactory bulb from neural stem cells. a) Frontal section of the brain that shows the subventricular zone along the lateral ventricles **b)** Sagittal section through the lateral ventricle that shows the larger area of adult neurogenesis, the subventricular zone (SVZ). Adult neurogenesis in the SVZ undergoes four developmental stages. Stage (1) Proliferation: stem cells *(red)* in the SVZ of the lateral ventricles give rise to transient amplifying cells *(green)*. Stage (2) Fate specification: transient amplifying cells differentiate into neuroblasts *(yellow)* Stage (3) Migration: migrating neurons migrate with each other in chains through the rostral migratory stream (RMS) to the olfactory bulb (OB). Once reaching the bulb, new neurons then migrate radially to the outer cell layers. Stage (4) Synaptic integration: migrating immature neurons differentiate into either periglomerular neurons or granule neurons. Adapted from Vescovi et.al., 2006

During the differentiation process, multipotent type-B astrocytes, which have been identified as the bona fide SVZ stem cells, give rise to fast-cycling transiently proliferating precursor cells that are called type-C precursors and that, in turn, generate mitotically active type-A neuroblasts. The type-A cells, while dividing, migrate towards the olfactory bulbs where they integrate as new interneurons (Ming and Song, 2005).

Within the subgranular zone of the hippocampal dentate gyrus (Fig. 5) neural stem cells give rise to transient amplifying cells (immature neuron) that migrate a short distance into the dentate gyrus granule cell layer. During the differentiation process, immature neurons extend their axonal projections to the CA3 pyramidal cell layer and their dendrites in the opposite directions toward the molecular layer to generate new granule neurons. These newly born granule neurons receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus region.

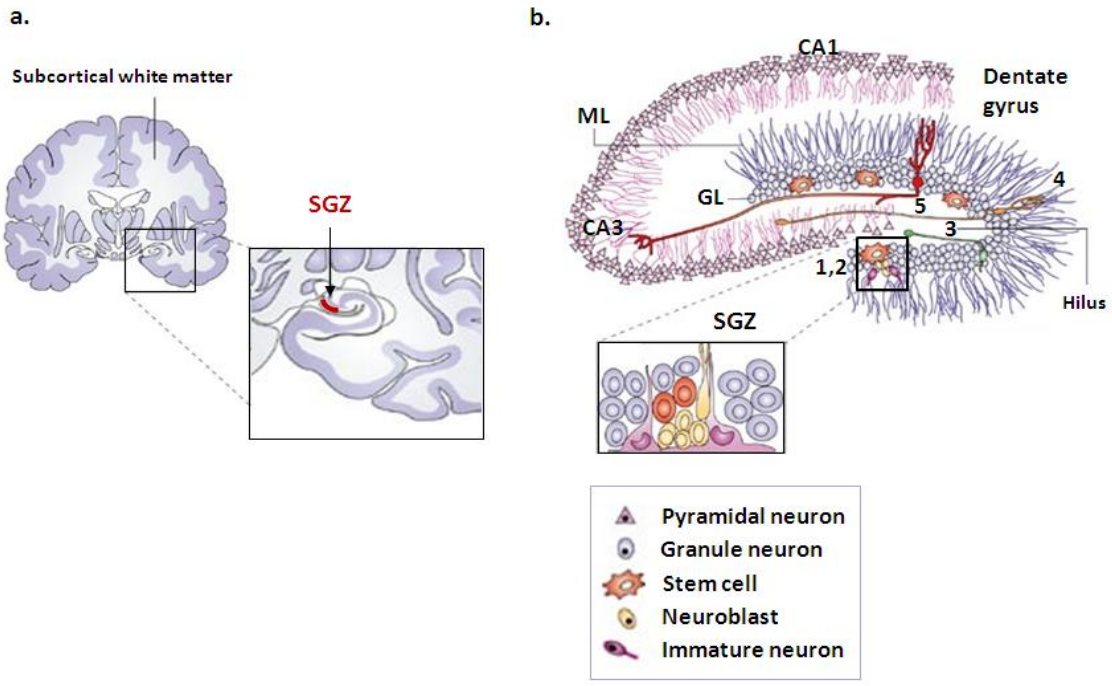

Figure 5 - Generation of new granular neurons in the dentate gyrus of the hippocampus from neural stem cells in the subgranular zone (SGZ) a) Frontal section of the brain that shows the SGZ in the dentate gyrus of the hippocampus. **b)** Sagittal section through the dentate gyrus. Adult neurogenesis in the dentate gyrus undergoes five developmental stages. Stage (1/2) Proliferation/Differentiation: Stem cells with their cell bodies within the SGZ give rise to transient amplifying cells that differentiate into immature neuron *(purple).* Stage (3) Migration: immature neurons *(green)* migrate a short distance into the granule cell layer. Stage (4) Axon Dendrite targeting: Immature neurons (orange) extend their axonal projections to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction toward the molecular layer (ML). Stage (5) Synaptic integration: New granule neurons *(red)* receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions. Adapted from Vescovi et.al, 2006

Since the discovery of neural stem cells and their involvement in adult neurogenesis, they have been intensively studied. However, their functional importance on brain activities has just begun to be revealed; and thus, further understanding of postdevelopmental neurogenesis is necessary to develop stem cell-based therapies for nervous system functional recovery, after disease, trauma or pathological aging(Imayoshi et al., 2011).

1.1.3 Induced pluripotent stem cells

Although the somatic stem cells possess a tissue-restricted range of differentiations options, they can be genetically reprogrammed to an embryonic stem cell-like state through the forced expression of key reprogramming transcription factors. One of the first studies involving somatic nuclear transfer (Gurdon et al., 1958) indicated that somatic cells can be reprogrammed to pluripotency.

However, the mechanisms and practical applications of inducing pluripotency in somatic cells have only become apparent in the last years (Watt and Driskell, 2010).

The original report (Takahashi and Yamanaka, 2006), demonstrated that retrovirus-mediated transduction of mouse fibroblasts with four transcription factors highly expressed in ESCs (Oct-3/4, Sox2, KLF4 and c-Myc) could induce the fibroblasts to become pluripotent. Since then, rapid progress has been made in these area and induced pluripotent stem cells (iPSCs) were generated from human somatic cells (Takahashi et al., 2007; Yu et al., 2007; Aasen et al., 2008; Park et al., 2008); cells from a range of tissues were reprogrammed (Aasen et al., 2008; Aoi et al., 2008) and iPSCs were generated from patients with specific diseases (Dimos et al., 2008; Park et al., 2008; Jang et al., 2012).

One of the attractions of transplanting iPSCs is that patient's own cells can be used, obviating the need for Immunosuppression (Watt and Driskell, 2010). Nevertheless, iPSCs technology is not yet ready for human trials. The current induced pluripotency protocols cannot efficiently eliminate residual and unwanted undifferentiated cells (Verma and Verma, 2011).

Furthermore, most patient-specific iPSCs have been generated using integrating vectors that could disrupt endogenous genes (Stadtfeld and Hochedlinger, 2010).

1.2 MAMMALIAN OLFACTORY SYSTEM

The olfactory system, which sits at the interface of the environment and the central nervous system, is responsible for discriminate and recognize thousands of chemical signals (odorous stimuli) presents in the environment. Therefore, this system regulates a wide range of multiple and integrative functions, such as emotional responses (e.g., anxiety, fear and pleasure), reproductive functions (e.g., sexual and maternal behaviors), and social behaviors (e.g., recognition of family, clan, or outsiders). To achieve this large varieties of functions, two anatomically and functionally separate sensory organs are required, the vomeronasal organ (VNO) and the main olfactory epithelium (MOE). In the VNO, sensory neurons project axons to the accessory olfactory bulb (AOB) and information provided by chemical compounds (i.e. pheromones) is then transmitted to the vomeronasal amygdala (VA) before reaching specific nuclei of the hypothalamus (H). Through this accessory olfactory system (Fig. 6), animals recognize evidences about the social and sexual status of other individuals (Lledo et al., 2005).

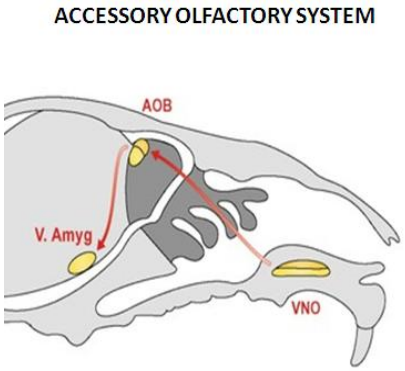

Figure 6 - Schematic sagittal view of the rat head that shows the accessory olfactory system. Axons of sensory neurons in the vomeronasal organ (VNO) project to the accessory olfactory bulb (AOB). Information is then transmitted to the vomeronasal amygdala (VA) before reaching specific nuclei of the hypothalamus. VNO, vomeronasal organ; AOB, accessory olfactory bulb; V.Amyg, vomeronasal amygdala. Adapted from Dulac and Torello, 2003

The human vomeronasal cavities can still be observed by endoscopy in some adults, but they lack sensory neurons and nerve fibers. In addition, no accessory olfactory bulbs are found and the genes which code for vomeronasal receptor proteins and the specific ionic channels involved in the transduction process are mutated and nonfunctional. Thus, the vomeronasal sensory function is nonoperational in humans (Trotier, 2011).

The second sensory organ is the main olfactory epithelium (MOE). This neuroepithelium is connected to the next central station for processing olfactory information: the main olfactory bulb (MOB). The output projections of the MOB target the primary olfactory cortex that include the anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the lateral part of the cortical amygdala (LA), and the entorhinal cortex (EC).

Through this main olfactory system (Fig. 7), animals recognizes more than a thousand different odorants (Lledo et al., 2005).

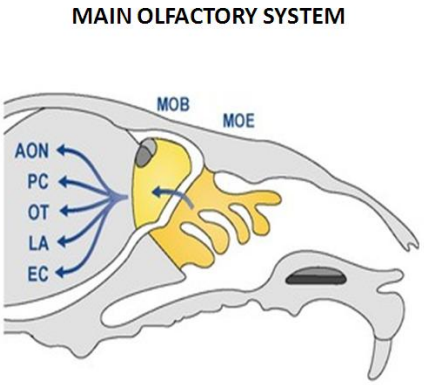

Figure 7 - **Schematic sagittal view of the rat head that shows the main olfactory system**. The presence of turbinates in the main olfactory epithelium (MOE) increases the surface area of the sensory organ. Axons of sensory neurons in the main olfactory epithelium (MOE) project to the main olfactory bulb (MOB). The output projections of the MOB target the primary olfactory cortex that include the anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the lateral part of the cortical amygdala (LA), and the entorhinal cortex (EC). Adapted from Dulac and Torello, 2003

1.2.1 Olfactory mucosa

The olfactory mucosa (Fig. 8) is the sense organ of smell and consists of two tissues - olfactory neuroepithelium and lamina propria – separated by a thin basement membrane.

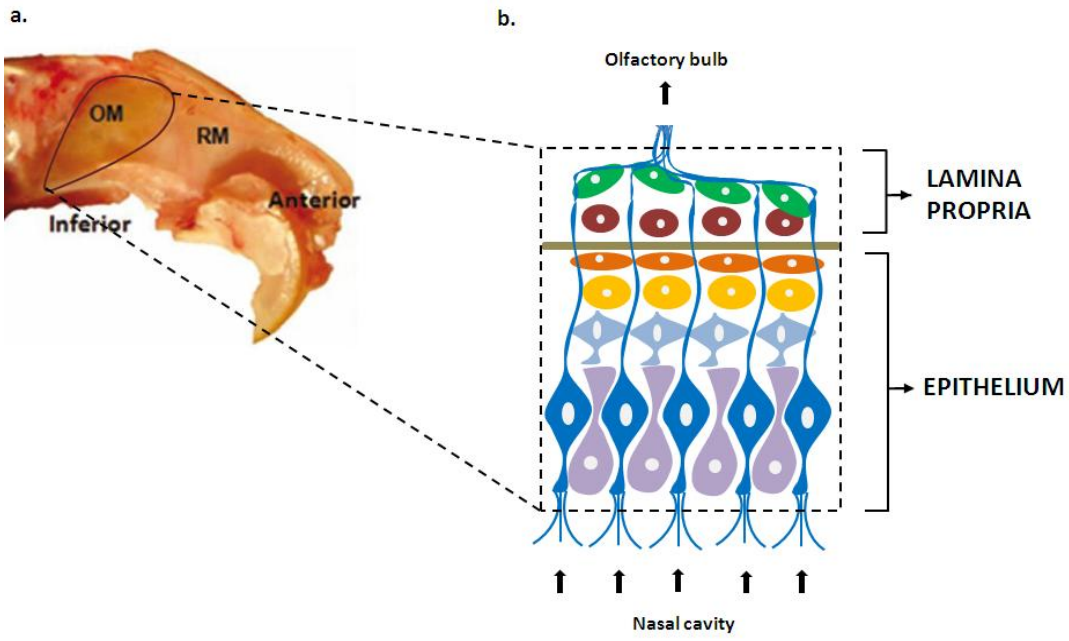

Figure 8 – a) Rat septum showing the position of the olfactory mucosa (OM). The yellow color of the OM allows distinguishing OM from respiratory mucosa (RM). **b) Schematic structure of the olfactory mucosa.** The olfactory mucosa is made up of two tissues - olfactory epithelium and olfactory lamina propria - separated by a thin basement membrane. Cellular composition of olfactory epithelium: olfactory receptor neurons *(blue),* sustentacular supporting cells *(purple)*, globose basal cells *(yellow)* and horizontal basal cells *(orange).* Cellular composition of lamina propria: olfactory ensheathing cells *(green),* ecto-mesenchymal stem cells *(maroon).* (a) Adapted from Tharion et al., 2011

In recent years, olfactory mucosa has attracted the interest of investigators as a potential marker for early diagnosis of neurological diseases. This growing interest emerged because the ability to identify and discriminate the odors, as well as the odor threshold can be altered in neurological diseases, such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, Huntington's disease and motor neuron disease (Escada et al., 2009; Barresi et al., 2012). Additionally, olfactory mucosa has achieved a particular attention since numerous studies reported the existence of stem cells niches in its composition (Mackay-Sim, 2010).

1.2.1.1 Olfactory Epithelium

The olfactory epithelium is the most superficial layer of the olfactory mucosa and is comprised of three main cell types: olfactory receptor neurons (ORNs), sustentacular supporting cells (SUS) and basal cells (Fig.8). At the surface of the epithelium, olfactory receptor neurons have a single dendrite that ends with a small knob (Wetzig, 2006). Projecting from this knob and embedded in the mucus layer are the olfactory non-motile cilia with membrane olfactory receptors where odor molecules bind (Escada et al., 2009). Olfactory receptors belong to the family of the G protein-coupled seven-transmembrane proteins that are encoded by the largest gene families known to exist in a given animal genome (Mombaerts, 1999; Mombaerts, 2001; Harkema et al., 2006).

The single axon of the olfactory receptor neurons passes through the basal lamina to join axons from other ORNs forming non-myelinated nerve fascicles, or bundles, in the lamina propria. These olfactory nerves perforate the boney cribriform plate, that separates the nasal cavity from the brain, and form the outer olfactory nerve layer of the olfactory bulb (Harkema et al., 2006). In the olfactory bulb, axons of the ORNs synapse within mitral cells and interneurons, some of which are generated by neurogenesis in the subventricular zone (SVZ) of the lateral ventricles (Mackay-Sim, 2010). The sustentacular supporting cells (SUS) surround olfactory receptor neurons, presumably contributing to regulating and maintaining the appropriate ionic milieu around the ORNs for olfactory transduction to occur.

Furthermore, there are known to be two distinct types of basal cells in the olfactory epithelium that do not project to the epithelial surface: horizontal basal cells (HBCs) and globose basal cells (GBCS) (Escada et al., 2009). HBCs are flat cells and lie along the basal lamina, whereas GBCs are round cells that are situated superficially to the HBCs.

1.2.1.2 Olfactory Lamina propria

The olfactory lamina propria is the tissue through which axons extend in order to synapse in the olfactory bulb. This mesenchymal tissue, lining the nasal cartilage, is composed of numerous blood vessels, nerve bundles, Bowman's glands and most of the cellular components of connective tissue (e.g., fibroblast, macrophages, leukocytes and mast cells) (Wetzig, 2006). In addition, lamina propria includes two type of cells: olfactory stem cells (OSCs) (Chuah and Au, 1991; Delorme et al., 2010; Girard et al., 2011) and olfactory ensheathing cell (OECs) (Wetzig, 2006). The OECs are specialized glial cells that guide the regeneration of non-myelinated olfactory axons from the peripheral nasal epithelium through the cribriform plate and into the olfactory bulb. By producing neurotrophic factors, neurite promoting factors and extracellular matrix molecules, OECs allow these axons to elongate and migrate (Guerout et al., 2011). Therefore, several studies have demonstrated the great potential of OECs to improve functional recovery and axonal re-growth after lesions of the central nervous system (spinal cord injury) or peripheral nervous system (peripheral nerves lesion) (Franssen et al., 2007; Radtke et al., 2009; Radtke and Vogt, 2009; Guerout et al., 2011; Tharion et al., 2011).

1.3 OLFACTORY MUCOSA-DERIVED STEM CELLS

1.3.1 Epithelium-derived stem cells: HBCs and GBCs

In 1940, it was first observed mitotic activity in the basal cells of the olfactory epithelium of adult mice (Nagahara, 1940) and soon after, regeneration of olfactory receptor neurons (ORNs) was observed in monkey after toxic damage (Schultz, 1941). Some years later, numerous reports confirmed this observation in frog (Smith, 1951), fish (Westerman and von Baumgarten, 1964), cat and dog (Andres, 1966), lamprey (Thornhill, 1970), mouse (Smart, 1971), monkey (Graziadei et al., 1980) and human (Wolozin et al., 1992; Murrell et al., 1996).

However, there was a long-running debate over the identity of the true olfactory stem cell population responsible for adult neurogenesis in the olfactory epithelium (Duggan and Ngai, 2007; Delorme et al., 2010; Mackay-Sim, 2010). While some studies have shown that the horizontal basal cells (HBCs) can generate neurons and glial cells in vitro (Feron et al., 1999; Carter et al., 2004), in parallel, convincing evidence demonstrated that globose basal cells (GBCs) give rise to neurons and sustentacular supporting cells (SUS) (Jang et al., 2003; Chen et al., 2004). Fortunately, this controversy over the identity of the neuroephithelioid stem cells came recently to a close (Delorme et al., 2010) with the publication of a study revealing that both basal cell types contribute to the neurogenic process and horizontal basal cells can fully reconstitute the olfactory neuroepithelium after an extensive injury (Leung et al., 2007). However, these cells exhibit a relatively poor proliferation rate in vitro and further investigation is required to improve their use either for comparative molecular studies or cell-based therapies (Wetzig et al., 2011).

1.3.2 Lamina propria-derived stem cells: OE-MSC

Since their discovery in (Tome et al., 2009), some studies have been focusing their attention in the lamina propria-derived olfactory stem cells (Delorme et al., 2010; Girard et al., 2011). Although their biological function remains to be revealed (Nivet et al., 2011), it is known that there is a strong association between these cells and the olfactory epithelium. Molecular signaling between lamina-propria and olfactory epithelium influences the olfactory pathway development (LaMantia et al., 2000); and during adulthood, cells migrating from one compartment to another can be observed, especially after an induced lesion. Hereupon, it can be surmised that the lamina-propria-derived stem cells are capable of crossing the basement membrane in order to differentiate into neurons and, possibly, replenish the olfactory epithelium after an extensive peripheral damage.

Delorme and co-workers have shown for the first time that lamina propria-derived stem cells can be considered as a mesenchymal cell subtype with specific traits associated to their location in an ectodermal tissue. Therefore, the open list of mesenchymal stem cells (MSCs) sources was updated with the inclusion of the lamina propria-derived stem cells, named as olfactory ecto-mesenchymal stem cells (OE-MSCs).

Transcript and membrane protein analyses have shown that OE-MSCs are closely related to bone marrow mesenchymal stem cells (BM-MSCs). When the expression of 21 cell surface proteins (know for portraying bone BM-MSCs) was assessed, only 3 markers were found differentially expressed in OE-MSCs. The CD9, that is consider a pluripotency marker (Hannan and Wolvetang, 2009) involved in cell motility, metastasis, osteoclastogenesis, neurite outgrowth, oligodendrogenesis, myotube formation, angiogenesis and cell proliferation is overexpressed; whereas CD146 and CD200 are clearly underexpressed in olfactory stem cells. Furthermore, when compared to BM-MSCs, lamina propria-derived stem cells also display a penchant for differentiating into cells with osseous phenotype. However, these cells rarely give rise to chondrocytes and, to a lesser extent, to adipocytes (Delorme et al., 2010).

Interestingly, OE-MSCs display a high proliferation rate *in vitro* (nearly 3-fold increase in population doubling time at week 2 post-plating) and were still able to proliferate in long-term cultures (over 15 weeks) whereas BM-MSCs failed to self-renew (Delorme et al., 2010). Additionally, when compared to BM-MSCs, lamina propria-derived stem cells display a high level expression of genes involved in neurogenesis, which clearly indicates that OE-MSCs are inclined to run along neural pathways (Delorme et al., 2010).

1.3.2.1 Isolation and proliferation of lamina-propria derived stem cells

Regarding the collection procedure, olfactory mucosa is easily accessible in every living individual and, even in humans, it can be safely collected by an ear, nose and throat (ENT) specialist under local anesthesia and without any loss of sense of smell (Feron et al., 1998; Girard et al., 2011).

Moreover, the procedure that follows to olfactory mucosa collection is relatively straightforward (Fig. 9) and is minutely described in a previous report (Girard et al., 2011).

Firstly, the olfactory lamina propria must be separated from the contiguous neuroepithelium by an enzymatic treatment (Dispase II enzyme; Fig. 9); and then, this purified tissue is dissociated by enzymatic (Collagenase IA enzyme; Fig. 9) and mechanic procedures (MP; Fig. 9) to release the OE-MSCs. Once established in a serum-containing culture medium, primary cell cultures rapidly proliferate as adherent monolayers (Girard et al., 2011).

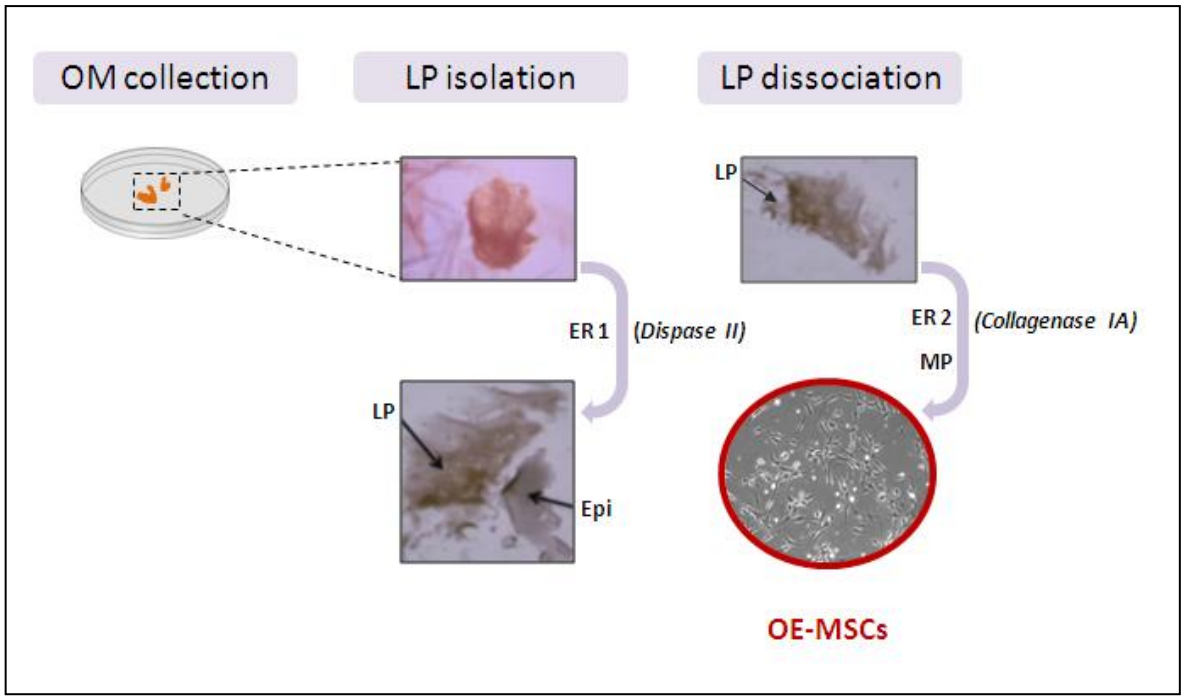

Figure 9 – Schematic procedure of olfactory ecto-mesenchymal stem cell (OE-MSCs) isolation from olfactory lamina-propria. OM, olfactory mucosa; LP, lamina propria; Epi, epithelium; ER, Enzymatic reaction; MP, mechanic procedure. Real Images Adapted from Girard et al., 2011

When grown in a serum-free culture medium supplemented with mitogens and on lysine substrate, olfactory stem cells give rise to three-dimensional spheres in suspension (Girard et al., 2011) (Fig. 10).

As well as the expression of stemness markers (i.e. Nestin), the neurospheres formation has also been extremely important during stem cells identification. OE-MSCs share this ability with stem cells from other neural tissues (e.g., spinal cord (Weiss et al., 1996), hippocampus (Shetty and Turner, 1998) and olfactory bulb (Ahn et al., 2008)) and from non-neuronal tissues (e.g., breast (Shackleton et al., 2006), heart (Tomita et al., 2005) and trachea (Rock et al., 2009)).

In non-neuronal tissues, the formation of spheres in culture was valuable in determining the ability of their stem cells to differentiate into neural cells types (Wetzig, 2006).

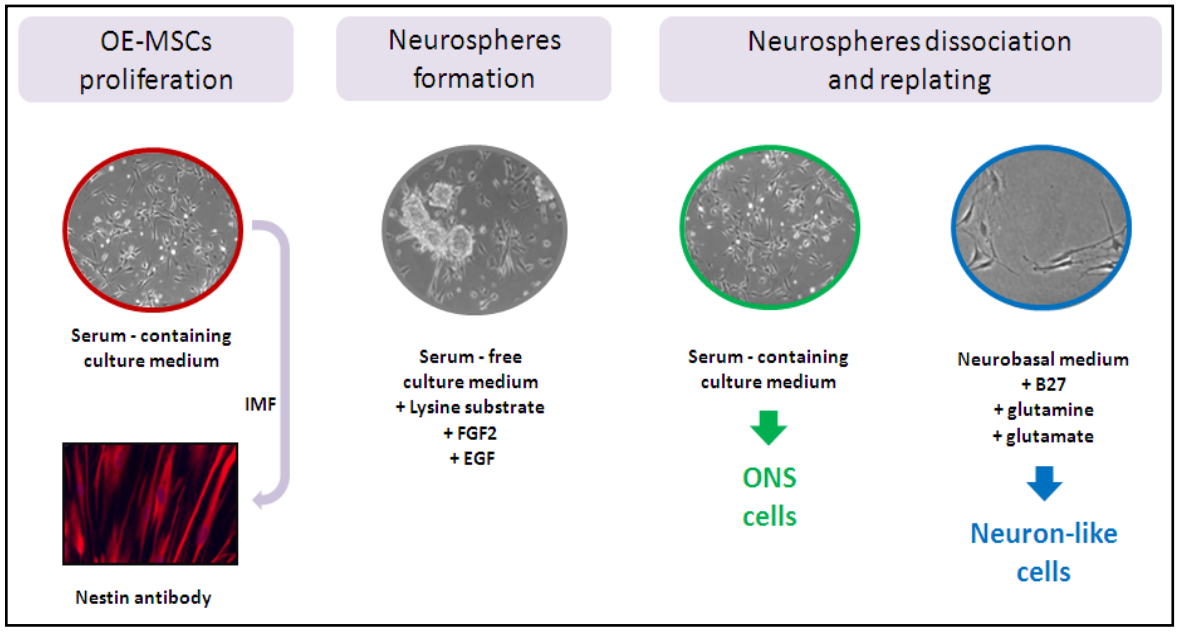

Figure 10 - Schematic procedure of the olfactory neurospheres-derived (ONS) cells and neuron-like cells generation. Olfactory ecto-mesenchymal stem cells (OE-MSCs) express the stemness marker nestin. IMF, immunofluorescence technique. Real Images Adapted from Girard et al., 2011

The growth factors that have been used to induce the highest number of neurosphere in OE-MSCs primary cultures are the epidermal growth factor (EGF) and the basic fibroblast growth factor (FGF2) (Girard et al., 2011); since they act as mitogens for neural stem cells and have been used in combination to expand neural stem cells (Kilpatrick and Bartlett, 1995; Weiss et al., 1996; Gritti et al., 1999). However, their role in determining the neural fate of OE-MSC is still unknown (Wetzig et al., 2011).

When the olfactory neurospheres reached about 100 µm in diameter they detached from the culture dish surface and becoming free-floating neurospheres (Matigian et al., 2010).

Then, these free-floating neurospheres should be collected, enzimatically or mechanically dissociated and re-plated. When the neurosphere are dissociated and re-plated in a serum-containing culture medium they give rise to olfactory neurospheres-derived (ONS) cells which growth as an adherent monolayer and exhibit some heterogeneity in their immunophenotype in culture (GFAP-expressing cells [~50%], β-tubulin III-expressing cells [~10-15%] and O4-expressing cells [~2-5%]). However, when the cells are re-plated in Neurobasal culture medium supplemented with B27, glutamine and glutamate their fate is modified and they differentiate into neuron-like cells expressing β-tubulin III and MAP2 (Girard et al., 2011).

1.3.2.2 Diagnostic and therapeutic uses for OE-MSCs

In the last 3 years, OE-MSCs have attracted the interest of investigators since they have taken advantage of their advantageous localization, high proliferation rate, ability to proliferate in long-term cultures, as well as their tendency to differentiate into neural cells; in order to perform comparative molecular studies or cell-based therapies for brain diseases (Girard et al., 2011).

With the evidence for olfactory mucosa alterations that depend from neurological diseases (including schizophrenia [SZ] (Brewer et al., 2003) and Parkinson´s disease [PD](Haehner et al., 2009)), a pioneering study by Matigian et al. 2010 hypothesized that the olfactory neurospheres-derived (ONS) cells from SZ and PD patients would exhibit disease-specific alterations. This comparative molecular study was performed using human ONS cells lines from patients (SZ, n=9; PD, n=19) and controls (control, n=14) and provided significant disease-specific alterations in gene expression, protein and cell function, namely dysregulated neurodevelopment pathways in SZ ONS cell lines and dysregulated mitochondrial function, oxidative stress and xenobiotic metabolism in PD ONS cell lines.

In a prior comparative molecular study performed using lymphocytes and skin fibroblasts (Matigian et al., 2008), the patient-control differences were modest both in SZ and PD, perhaps because these cell types do not reflect tissue-specific differences important for brain function (Wang et al., 2010).

These evidences revealed that cells collected from primary sources that have been subjected to signals appropriate for the pathological specificity are crucial to mirror the disease human neural cells (Boone et al., 2010). Therefore, human ONS cells lines provide a neural tissue-specific cellular model for neurological diseases, which can be easily accesible from patients, grown in standardized conditions, frozen, banked, thawed, and re-grown in quantity for gene and protein expression analyses, functional studies and high-throughput drug screening (Matigian et al., 2010).

So far, human OE-MSCs allowed to unveil new candidate genes and cell pathways involved in schizophrenia (Matigian et al., 2010), Parkinson's disease (Matigian et al., 2010; Cook et al., 2011), and familial dysautonomia (Boone et al., 2010). However, it is important to note that, since ONS cells exhibit a relatively undifferentiated state, the disease-related differences might be magnified or altered if these cells were differentiated into the relevant cells of the nervous system (Matigian et al., 2010). Thus, there is an urgent need for comparative molecular studies using both ONS cells lines and olfactory neurospheres-derived differentiated neural cells.

In the National Centre for Adult Stem Cell Research (Griffith University; Australia), frozen aliquots of ONS cells from healthy controls and patients with nervous system disorders (including Parkinson's disease, schizophrenia, motor neuron disease, hereditary spastic paraplegia, multiple sclerosis and mitochondrial mutation disease) are available; subject to patient consent, an Appropriate Material Agreement, and payment of shipping and handling fees. Hereupon, these patient-derived, disease-specific cell lines can be compared to identify the gene networks and biochemical pathways that contribute to disease and to investigate gene-environment interactions. Furthermore, these cell lines can also be used in the drug development process for assessing the toxicity of new drug candidates and for assessing their efficacy.

In addition to its value as a starting point to establish a stem cell based model system to study neuropathologies, some studies have also reported the value of the OE-MSCs as a promising candidate for stem cell-based therapies. A previous study reported that the transplantation of adult human OE-MSCs into the cochleae of a mouse model of early-onset sensorineural hearing loss, contribute to a reduction in hearing loss when compared with sham-treated animals (Pandit et al., 2011). In parallel, a second report demonstrates that the engraftment of human OE-MSCs into mouse damaged hippocampus also holds therapeutic value; since the exogenous stem cells migrate toward the inflamed areas, exhibit in situ neuronal differentiation, stimulate endogenous neurogenesis, restore defective learning and memory abilities, and enhance physiological function (i.e., long-term potentiation [LTP])(Nivet et al., 2011).

2. AIMS OF THE DISSERTATION

The possibility to investigate neuropathologies using stem cells based models systems has been widely explored in the last years. Recently, lamina propria-derived stem cells from olfactory mucosa have attracted interest among the scientific community. The innovation of this novel model is the advantage of easily accessible location, high proliferation rate, ability to proliferate in long-term cultures and tendency to differentiate into neural cells. Nonetheless, the main reason for the upcoming interest in this specific cell type is their ability to demonstrate disease-related differences in gene expression, protein expression and cell function (i.e.: schizophrenia, Parkinson's disease, and familial dysautonomia).

Therefore, the main objectives of this dissertation were:

- Isolation and proliferation of olfactory stem cell from rat olfactory mucosa;
- Differentiation of olfactory stem cells into olfactory neurospheres derived cells (ONS) and neuron like cell (NLC);
- Characterization of olfactory neurospheres-derived cells (ONS) model system.

The methodology used to achieve these goals was based on very recent publication of Girard and co-workers.

3. MATERIALS AND METHODS

3.1 ISOLATION AND PROLIFERATION OF OLFACTORY MUCOSA-DERIVED STEM CELLS

3.1.1 Isolation of olfactory mucosa from rats

Olfactory mucosa was obtained from Wistar female rats (9-15 weeks) handled according to the European Union guidelines (86/609/EEC). These animals were obtained from IBMC (Instituto de Biologia Molecular e Celular) and housed under controlled environment (26°C under a 12 hour light/dark cycle) with food and water available *ad libitum*.

Animals were sacrificed by rapid cervical stretching followed by decapitation and skin removal. Facial muscles were eliminated on both sides and the lower jaw was removed with the help of scissors and a rongeur. Then, the bone that covered one of the nasal cavities was removed starting from the back incisors. When the olfactory turbinates appeared in the back of the nose, they were carefully tweezed and the olfactory mucosa was exposed. Most of the olfactory mucosa was transferred to a Petri dish filled with culture medium (DMEM/HAM F12).

In order to collect olfactory mucosa lysates, a short fragment of 2 mm x 2 mm was transferred to a microtube and 1ml of culture medium was added. The sample was centrifuged at 1000 rpm for 3 minutes and the supernatant removed and olfactory mucosa resuspended in 150µl of boiling 1% SDS. The sample was further sonicated five times during 10 seconds, boiled during 10 minutes and then stored at -20°C. Protein content of the sample was determined using the BCA assay (as described below).

3.1.2 Establishment of primary stem cell cultures from olfactory mucosa

Olfactory mucosa from both sides were washed three times in DMEM/HAM F12 and then incubated in a 35 mm cell Petri dish with 1ml of dispase II solution (2.4 IU/ml), for 1 hour at 37°C (Fig. 11).

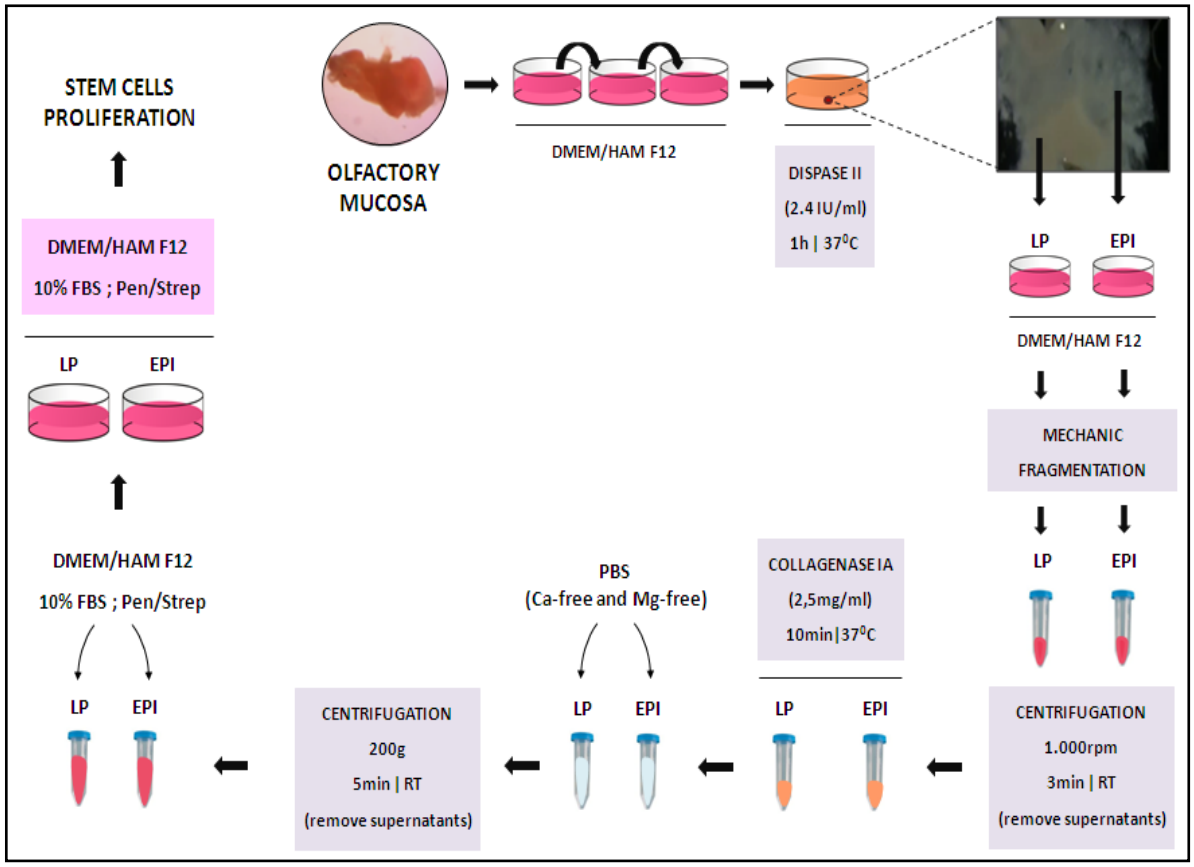

Figure 11 – Overall scheme of the experimental procedure to establish primary stem cells cultures from olfactory mucosa. LP: lamina propria; EPI: Epithelium. Real images from (Girard et al., 2011)Girard et al., 2011

Then, under a dissecting microscope with a diffracted inverted light and over a black background, the lamina propria was carefully separated from the underlying olfactory epithelium. The lamina propria, that seemed striped orange/brown, was tweezed with the help of a micro spatula to a new culture dish filled with DMEM/HAM F12; while the thinner and translucent epithelium was transferred to a second culture dish also filled with DMEM/HAM F12 (Fig. 12).

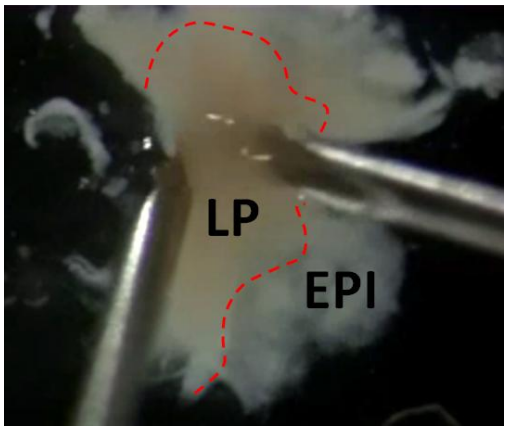

Figure 12 – Olfactory mucosa under a dissection microscope. The putative barrier between lamina propria (LP) and epithelium (EPI) is depicted *(red dashed line).* Adapted from Girard at al., 2011.

Using two 25 gauge needles, lamina propria and epithelium were fragmented into small pieces, and then the culture medium with the floating fragments was transferred to 15 ml tubes. The tubes were then centrifuged at 1000 rpm for 3 minutes at room temperature (RT). Next, the supernatants were discarded and 1ml of collagenase IA (2,5mg/ml) added to each pellet (Fig. 11). The pellets were then dissociated using a sterile plastic pipette and incubated with collagenase IA solution for 10 minutes at 37°C. To terminate the dissociation, the tubes were gently rocked and 9ml of Ca-free and Mg-free PBS was added in each tube. The tubes were then centrifuged at 1000 rpm for 5 minutes and the supernatants were discarded. The cell pellets in each tube were resuspended in 2ml of DMEM/HAM F12 culture medium (supplemented with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin [Pen/Strep] solution) and then plated on 35 mm cell culture petri dishes. All cultures were grown under standard conditions at 37°C and 5% CO_2 and for either tissue type the culture medium was totally renewed every 2 days.

3.1.3 Propagation of stem cells

Nearly one month after the establishment of the primary cell cultures in 35 mm plastic culture dishes, stem cells were confluents. At this time, the culture medium was aspirated and cells were washed with PBS (1X). Then, the cells were incubated with 1ml of trypsin-EDTA solution for 3 minutes at 37°C. To stop the trypsin action, 2ml of culture medium supplemented with FBS (DMEM/HAM F12 supplemented with 10% FBS and 1% Pen/Strep) were added to culture dishes. Culture medium with the resuspended stem cells was then transferred to 15ml tubes that were centrifuged at 1000 rpm for 3 minutes. After the removal of supernatants the cell pellets were resuspended in 2ml of culture medium supplemented with FBS.

Then, the cell suspensions were equally distributed for 2 plastic culture dishes (60 mm) that were filled with 3ml of serum-containing culture medium. Whenever the cells reached the confluence state, this cells passage procedure was repeated.

In order to collect stem cells lysates, cells from confluents 60mm cell culture petri dishes were detached with trypsin and then centrifuged at 1000 rpm for 3 minutes. Then, the supernatants were removed and the cell pellets resuspended in 1 ml of serum-containing culture medium. The cell suspensions were transferred to microtubes and centrifuged at 300g for 3minutes. The supernatants were removed and then 150µl of boiling 1% SDS were added to the microtubes that were immediately placed on ice. The samples in the microtubes were sonicated five times during 10 seconds, boiled during 10 minutes and then stored at -20°C. Protein content in the samples was determined using the BCA assay (as described below).

3.2 OLFACTORY NEUROSPHERES FORMATION AND GROWING

Primary cultures from olfactory lamina propria and epithelium were expanded as a monolayer in culture medium supplemented with FBS. To form neurospheres, the culture medium was aspirated and cells were washed with PBS (1X).

Then, the cells were incubated with trypsin-EDTA solution for 3 minutes at 37°C. To stop the trypsin action, culture medium supplemented with FBS was added to culture petri dishes. Culture medium with the resuspended stem cells was then transferred to 15ml tubes that were centrifuged at 1000 rpm for 3 minutes. After the removal of supernatants the cell pellets were resuspended in serum-free culture medium (DMEM/HAM F12 supplemented with insulin, transferrin, selenium [ITS-X 1%]; EGF [50ng/ml]; FGF2 [50ng/ml] and 1% Pen/Strep) and plated on poly-D-lysine-coated 60mm cell culture petri dishes. Every two days, half of the medium was changed.

In order to collect olfactory neurospheres lysates, the culture medium with the floating neurospheres was transferred to 15ml tubes (first step). Then, 2ml of serum-free culture medium were added to the dishes and with a micropipette, fluxes and refluxes were performed to release the neurospheres that were still adherents. This neurospheres in suspension were added to the tubes that were used in the first step. The tubes were centrifuged at 1000 rpm for 3 minutes, the supernatants were removed and the cell pellets resuspended in 1 ml of serum-free culture medium. The cell suspensions were transferred to microtubes and centrifuged at 300g for 3minutes. The supernatants were removed and then 150µl of boiling 1% SDS were added to the microtubes that were immediately placed on ice. The samples in the microtubes were sonicated five times during 10 seconds, boiled during 10 minutes and then stored at -20°C. Protein content in the samples was determined using the BCA assay (as described below).

3.3 ONS CELLS FORMATION AND PROLIFERATION

Culture medium with the floating neurospheres was transferred to 15ml tubes (first step) and then, 2ml of serum-free culture medium were added to the dishes and with a micropipette, fluxes and refluxes were performed to release the neurospheres that were still adherents. This neurospheres in suspension were added to the tubes that were used in the first step. The tubes were centrifuged at 1000 rpm for 3 minutes and the supernatants were removed.

Then, the cell pellets were resuspended in 1 ml of trypsin and incubated for 1 minute at 37°C. Culture medium supplemented with FBS was added to the tubes to stop the trypsin action and then, the tubes were centrifuged at 1000 rpm for 3 minutes. After the removal of supernatants, the cell pellets were resuspended in 2ml of serum-containing culture medium. Then, the cell suspensions were re-plated on poly-D-lysine coated 60mm cell culture petri dishes that were filled with 2ml of serum-containing culture medium. Olfactory neurospheres-derived (ONS) cells were grown under standard conditions at 37^0C and 5% CO_2 the culture medium was totally renewed every 2 days. Whenever the cells reached the confluence state, the cells passage procedure described in section 3.1.3 was repeated.

In order to collect ONS cell lysates, cells from confluents were detached with trypsin and then centrifuged at 1000 rpm for 3 minutes. Then, the supernatants were removed and the cell pellets resuspended in 1 ml of serum-containing culture medium. The cell suspensions were transferred to microtubes and centrifuged at 300g for 3minutes. The supernatants were removed and then 150µl of boiling 1% SDS were added to the microtubes that were immediately placed on ice. The samples in the microtubes were sonicated five times during 10 seconds, boiled during 10 minutes and then stored at -20°C. Protein content in the samples was determined using the BCA assay (as described below).

3.4 NEURON-LIKE CELLS FORMATION AND PROLIFERATION

Culture medium with the floating neurospheres was transferred to 15ml tubes (first step) and then, 2ml of serum-free culture medium were added to the dishes and with a micropipette, fluxes and refluxes were performed to release the neurospheres that were still adherents. This neurospheres in suspension were added to the tubes that were used in the first step. The tubes were centrifuged at 1000 rpm for 3 minutes and the supernatants were removed. Then, the cell pellets were resuspended in 1 ml of trypsin and incubated for 1 minute at 37°C.

Culture medium supplemented with FBS was added to the tubes to stop the trypsin action and then, the tubes were centrifuged at 1000 rpm for 3 minutes. After the removal of supernatants, the cell pellets were resuspended in Neurobasal medium (Gibco), containing B-27 (1X), glutamine (2mM), glutamate (0,025mM), 1% Pen/Strep and phenol red; and then plated on poly-D-lysine-coated 60 mm cell culture petri dishes. All cultures were grown under standard conditions at 37^0C and 5% CO_2 and half of the medium was changed every 2-3 days.

3.5 IMMUNOCYTOCHEMISTRY

Cells grown on poly-D-lysine-coated coverslips were fixed with a 4% paraformaldehyde solution. Thus, the culture medium was aspirated and then, 1,5ml of serum-containing culture medium and 1,5ml of 4% paraformaldehyd solution were added to the dish. After 10 minutes, the medium/paraformaldehyde solution was aspirated and 3ml of 4% paraformaldehyde solution were added to the dish for 20 minutes. After that period the cells were washed 5 times with PBS and permeabilized with a 0,2% Triton solution during 2 minutes. After permeabilization, cells were washed 5 times and then blocked with a solution of 3% BSA in PBS solution for 1 hour.

The cells were then incubated with the primary antibodies diluted in 3% BSA in PBS (the adequate dilutions are present in Table. 1) for 4 hours at room temperature.

The primary antibodies were removed by washing 3 times with PBS and the specific secondary antibodies were added for 2hours at room temperature (see Table. 1). After 3 washes with PBS and one with deionised water, the cells were mounted with VECTASHIELD® mounting medium with DAPI.

Table 1: Primary and secondary antibodies used for detection of nestin and β-Tubulin III. Specific immunocytochemistry dilutions are indicated of both primary and secondary antibodies.

Target Protein	Primary antibody	Secondary antibody
Nestin	Monoclonal Rat (MAB-353; Millipore) Dilution (1:100)	Texas Red®-X goat anti-mouse IgG (LifeTechnologies) Dilution (1:300)
β-Tubulin III	Monoclonal Rat (MAB-1637; Millipore) Dilution (1:500)	Texas Red®-X goat anti-mouse IgG (LifeTechnologies) Dilution (1:300)

3.6 BCA PROTEIN QUANTIFICATION ASSAY

The bicinchoninic acid (BCA) assay (Pierce) is a biochemical assay used to determine total protein content in a test sample. This method consists in a color change of the sample solution from green to purple in proportion to protein concentration. This assay is performed in two reactions. Firstly, Cu^{2+} is reduced to Cu^{+} by protein in an alkaline medium, which results in a light blue complex - biuret reaction (temperature dependent reaction). This reaction occurs with high sensitivity. Then, the colorimetric detection is allowed through the colorimetric detection of Cu^{+} cation, that was formed in step one, by BCA. More precisely, the reaction of two molecules of BCA with one Cu+ ion, results in an intense purple-colored reaction product. The BCA/Cu^{+} complex is water-soluble and exhibits a strong linear absorbance at 562nm with increasing protein concentrations (over a working range between 20µg/ml to 2000 µg/ml).

The standards were prepared in duplicate as described in Table 2. The final volume for each standard and sample was equal to 25µl.

Table 2: Summary of BCA standards preparations. BSA, bovine serum albumin; SDS, Sodium dodecyl sulfate

Standards	BSA (µl)	1% SDS (µl)	Protein mass (µg)
P0	0	25	0
P1	1	24	2
P2	2	23	4
P3	5	20	10
P4	10	15	20
P5	20	5	40

After prepare BCA standards, the samples were prepared also in duplicate, using 5µl of the collected lysates and 20 µl of 1% SDS (final volume of 25 µl). Then, 200 µl of Working reagent (WR) were added to both standards and samples. WR was prepared adding 50 parts of reagent A to 1 part of reagent B. After that, the microplate was stirred slowly for 1min at RT and incubated at 37^0C during 30 minutes. Then, the plate were cooled at room temperature during 2 minutes and the absorbances were measured at 562 nm using the microplate reader Infinite® M200 (Tecan) and the i-control ^{TM} software.

A standard curve was prepared by plotting the optical density (OD) value for each BCA standard against its concentration, which allowed the determination of protein concentration of each sample.

3.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) AND ELECTROTRANSFERENCE

Sodium dodecyl sulfate – polyacrilamide gel electrophoresis (SDS-PAGE) is an analytical technique commonly used to separate proteins based on their molecular weight and negative net charge due to SDS-amino acid binding. SDS is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone, which results in an unfolding and individual polypepetide.

This reaction occurs in presence of SDS when the samples are heated to 100°C. SDS-PAGE can thus be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. The migration of heavy molecules is slower, while the migration of light molecules is fast.

After preparing the samples, they were subjected to a 5% - 20% gradient SDS-PAGE. First, gradient gels were prepared and then allowed to polymerize between two glasses plates for 45 minutes at room temperature. Subsequently, the stacking gel solution was prepared and loaded on the top of gradient gel. Then, a comb was inserted and the gel was left to polymerize for at least 30 minutes at RT.

During the polymerization time, the samples were prepared by adding Loading buffer (LB) to each tube. Then they were boiled for 10 minutes and a spin down was done to each sample. After that, the samples were carefully loaded into the wells. A molecular weight marker was also loaded (Kaleidoscope Prestained Standards and Dual Colour Prestained Standards – Broad range, Bio Rad) and resolved side-by-side with the samples. Gels were run at 90 mA during approximately 3 hours. After running the proteins, they were electrotransferred to a solid support (nitrocellulose membranes, Whatman®) for 18 hours at 200 mA. After electrotransference the detection of the protein of interest was carried out by immunoblotting (as described below).

3.8 IMMUNOBLOTTING

During the electrotransference, transferred proteins become immobilized on the surface of the nitrocellulose membrane in a pattern that is an exact replica of the gel. After that, the detection of proteins is carried out using specific antibodies (primary antibodies) against the proteins of interest. The following primary antibodies were used: rabbit polyclonal C-TERM antibody (Invitrogen), which detects all APP isoforms (holo-APP), mouse monoclonal Tau-5 antibody (Millipore) to detect all phosphorylated and non phosphorylated isoforms of Tau (total-Tau) and mouse monoclonal antibody (Millipore) against Nestin (Table 3.).

Table 3: Summary of primary antibodies used, as well, the respective target, isotype, dilution and the expected band size

Antibody	Target	Isotype	Dilution	Expected band size (KDa)
C-TERM	holo-APP	Rabbit (polyclonal)	1:1000	100 - 140
Tau - 5	total-tau	Mouse (monoclonal)	1:500	56 - 68
Nestin	Nestin	Mouse (monoclonal)	1:1000	200-220

As secondary antibodies, Horseradish peroxidase anti-rabbit and anti-mouse (GE Healthcare) were used both with a dilution of 1:5000.

Once the immunoblotting protocol is antibody specific, the protocols used were summarized in the Table 4.

Immunoblotting was performed by initially soaking the membranes in 1X TBS for 5 minutes and then blocking non-specific binding sites of the primary antibody by incubating the membrane with 5% non-fat dry milk/5% BSA in 1x TBS-T (during 2 or 4 hours). This step allows that unoccupied protein binding sites on the membrane were saturated to prevent non-specific binding of antibodies.

Then, the membranes were further incubated with the specific primary antibody solution (previously prepared according to the respective dilution), during 2 or 4 hours with agitation and then sit overnight at 4°C (Table. 4). After the incubation period, membranes were washed with 1x TBS-T; incubated with secondary antibody during 2 hours under agitation at room temperature. The secondary antibody solution (specific for the primary antibody) was used at 1:5000 and is diluted in 3% non-fat dry milk in 1x TBS-T or 3% BSA in 1X TBS-T. Membranes were then washed three times with 1X TBS-T, during 10 minutes each.

Table 4: General immunoblotting protocol used for each antibody. ON, overnight; RT, Room Temperature; min, minutes; h, hours

Antibody	Hydration	Blocking agent	Primary antibody	Washings	Secondary antibody	Washings	Detection Method
C-TERM	-1x TBS -5 min	- 5% low fat milk in 1x TBS-T -4h at RT	-3% BSA in 1x TBS-T; -4h at RT + ON at 4°C	-1x TBS-T; -3times; -10min each	-3% BSA in 1x TBS-T; -2 h at RT	-1x TBS-T; -3times; -10min each	ECL
Tau 5	-1x TBS -5 min	-5% BSA in 1x TBST; -2h at RT	-3% BSA in 1x TBS-T; -2h at RT	-1x TBS-T; -3times; -10min each	-3% BSA in 1x TBS-T; -2h at RT	-1x TBS-T; -3times; -10min each	ECL
Nestin	-1x TBS -5 min	- 5% low fat milk in 1x TBS-T; -2h at RT	-3% low fat dry milk in 1x TBS-T; -2h at RT	-1x TBS-T; -3times; -10min each	-3% low fat dry milk in 1x TBS-T; -2h at RT	-1x TBS-T; -3times; -10min each	ECL

Then, the membranes were incubated for 1 minute at RT with ECL detection kit (GE Healthcare) in a dark room and then placed on an x-ray film cassette with a sheet of film on top of it.

The cassette was closed during an appropriate time to obtain signal and then the film was developed and fixed with appropriate solutions. Films were dried and quantified subsequently.

3.7 QUANTIFICATION

Quantitative analysis of immunoblots was performed using the Quantity One GS-800 densitometry software (Bio-Rad). This software allows the quantifications of band intensity and correlates it to protein levels.

4. RESULTS

4.1 ISOLATION AND PROLIFERATION OF OLFACTORY STEM CELLS FROM RAT OLFACTORY MUCOSA

The experimental procedure to isolate and establish primary cell cultures of olfactory stem cells from rat olfactory mucosa is still being optimized for several research groups. In our laboratory this procedure had never been made before and all the experiments were based mainly on a study published very recently (Girard et al., 2011) that presented a detailed protocol to isolate, proliferate and differentiate olfactory stem cells.

To isolate and establish primary cell cultures from rat olfactory mucosa, two animals were sacrificed (named Rat 1 and Rat 2). The olfactory mucosa from both rats was dissected out as described in the section 3.1.1.

Briefly, the nasal bone (N.B) was removed and then, the olfactory turbinates (O.T) appeared in the back of the nose (Fig. 13). The olfactory turbinates easily come into sight as orange/brown organs however they must be carefully tweezed because the underlying olfactory mucosa (O.M) tends to cling to the olfactory turbinates, leading to an undesirable loss of biological material. The exposed olfactory mucosa (Fig.13) was then transferred to a 35 mm Petri dish filled with culture medium (DMEM/HAM F12).

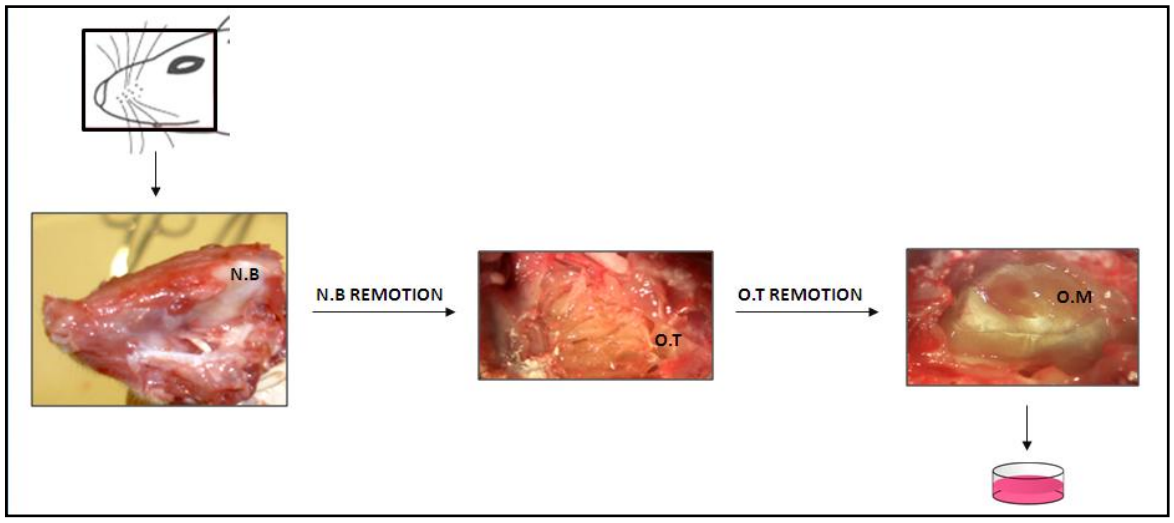

Figure 13 – Outline of the experimental protocol to isolate olfactory mucosa from rats. N.B: nasal bone; O.T: olfactory turbinates and O.M: olfactory mucosa. Real images from O.T and O.M adapted from Girard at al., 2011.

After washing, the olfactory mucosa was incubated with dispase II solution (2,4U/ml) for 1 hour at 37°C. Next, under a dissecting microscope and over a black background, the lamina propria (LP) was separated from the contiguous olfactory ephitelium (EPI) based on their color and thickness (Fig. 14). Compared to lamina propria, which is striped orange/brown, the olfactory epithelium is thinner and looks white or translucent (Fig. 14).

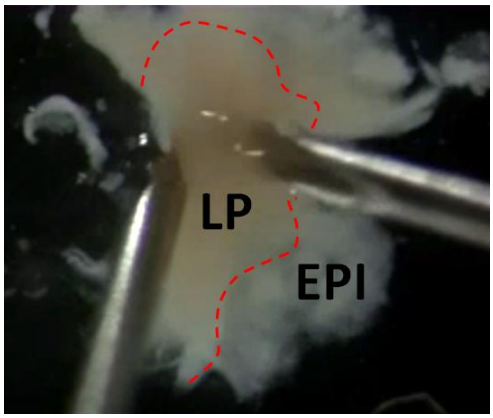

Figure 14 – Olfactory mucosa under a dissection microscope. The putative barrier between lamina propria (LP) and epithelium (EPI) is depicted *(red dashed line).* Adapted from Girard at al., 2011.

After LP and EPI preparation they were both dissociated for 10 minutes at 37°C in collagenase IA solution (2,5mg/ml) and plated on 35 mm cell culture petri dishes filled with DMEM/HAM F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Pen/Strep).

All cultures were grown under standard conditions at 37°C and 5% CO_2 to propagate any potential stem cell populations.

For both tissue type (LP and EPI) the culture medium was totally renewed every 2 days and divided when they were 90% confluents. Passage number (PN) and their respective day in culture were always registered over the time and the results are presented in Figure 15.

We can observe that primary cultures established from rat 1 were subject to 9 passages and remained in culture during 50 (LP-derived cells) and 59 days (EPI-derived cells); while primary cultures from rat 2 were subject to 6 passages and remained in culture during 30 (lamina propria-derived cells) and 43 days (epithelium-derived cells).

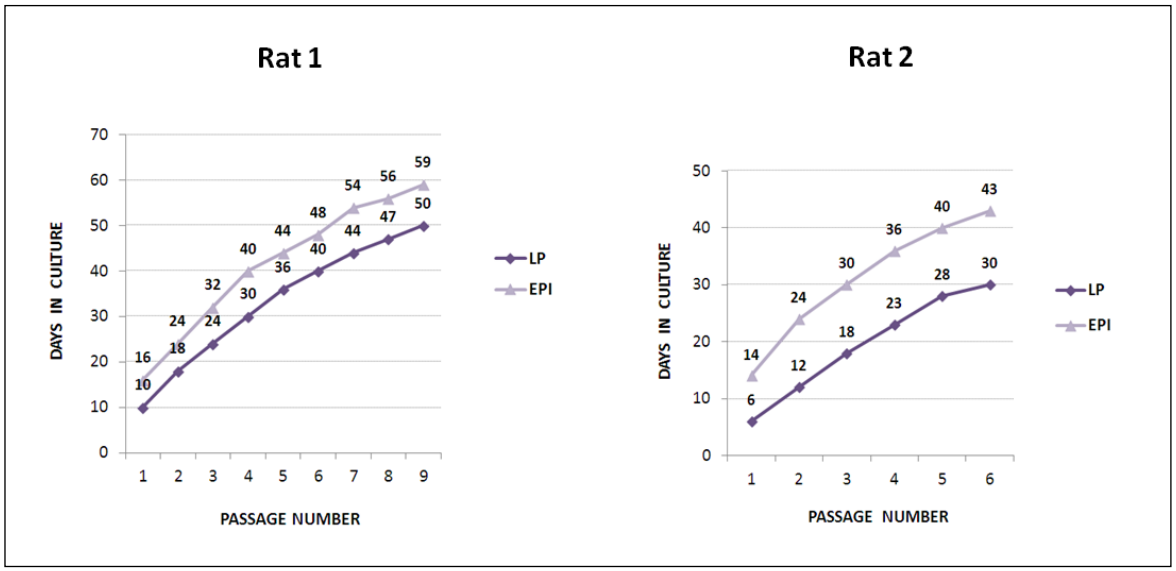

Figure 15 – Passage number and respective days in culture for both primary cultures isolated from LP and EPI. LP: lamina propria, EPI: epithelium

The confirmation that the isolation procedure was achieved and that the primary cells cultures from LP and EPI are proliferating, their morphological appearance was observed. These cells have been previously described as elongated cells that proliferate as adherent monolayers (Delorme et al., 2010). Thus, to examine these cells, phase-contrast microphotographs were taken on primary cultures established from rats 1 and 2 at several passages (PN) and the results presented in Figure 16. Primary cultures were composed mainly of elongated adherent cells and there were no significant morphological differences between lamina propria and epithelium derived olfactory cells. Additionally, we could observe that their elongated morphology, as well as their ability to grow as adherent monolayers, was maintained during the time course.

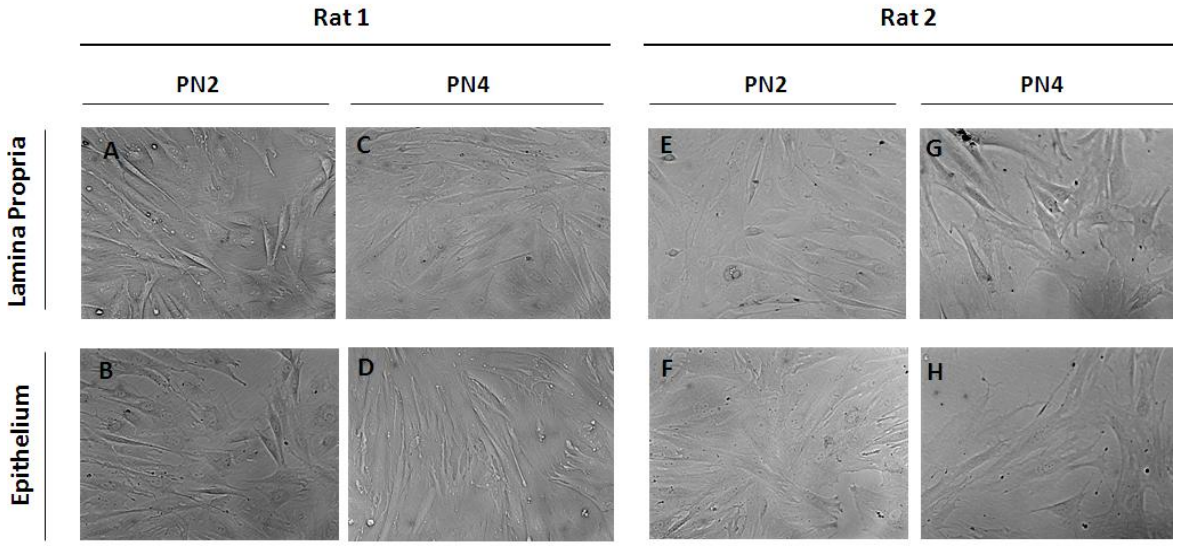

Figure 16 – Morphological examination of stem cells morphology. Phase-contrast microphotographs taken from primary cultures isolated from rat 1 *(left)* and rat 2 *(right*)**.** For each type of culture (Lamina propria and Epithelium), microscopic examination was done during time course and the results presented after passage number 2 (A, B and E, F) and 4 (C, D and G, H). PN: passage number

Although the morphological analyses of LP and EPI derived cultures suggests that we successfully isolated and established primary cell cultures from rat olfactory mucosa, we had to verify whether stem cells were present in these primary cell cultures. Thus, lamina propria and epithelium-derived cells from rats 1 and 2 were immunolabeled with anti-nestin monoclonal antibody (stemness marker), while their nuclei were labeled with DAPI, and the results are presented in Figure 17.

From the immunofluorescence images obtained, we could observe that primary cultures from rats 1 (Fig. 17) and rat 2 (Fig. 18) expressed the stemness marker nestin; either in lamina propria (Fig. 17; A, D and Fig. 18; A, D) or epithelium derived cultures (Fig. 17; G, J and Fig. 18; G, J).

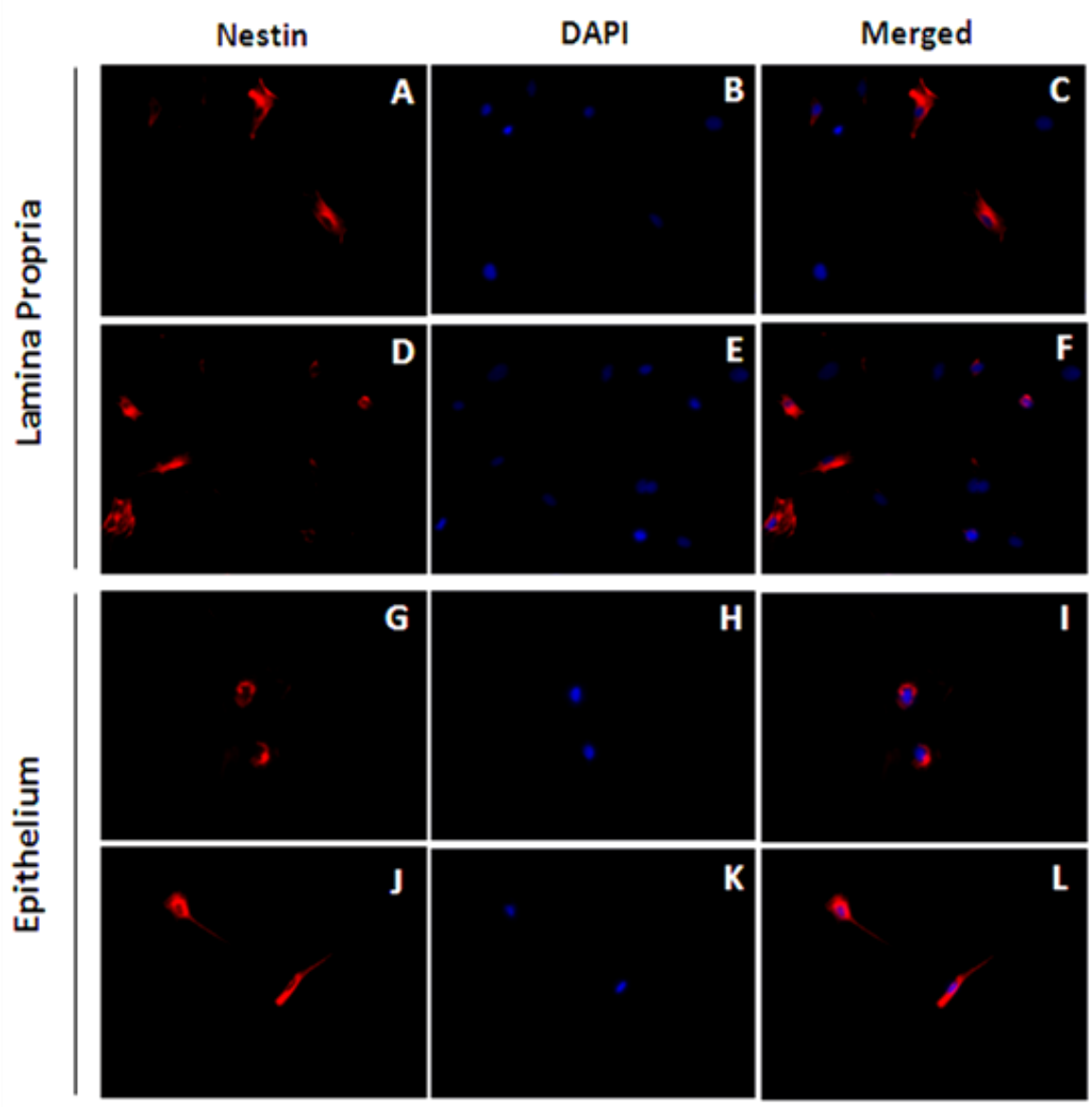

Figure 17 - Nestin immunolocalization in lamina propria (A, D,) and epithelium derived stem cells (G, J) isolated from rat 1. Cell nuclei were simultaneously stained with DAPI.

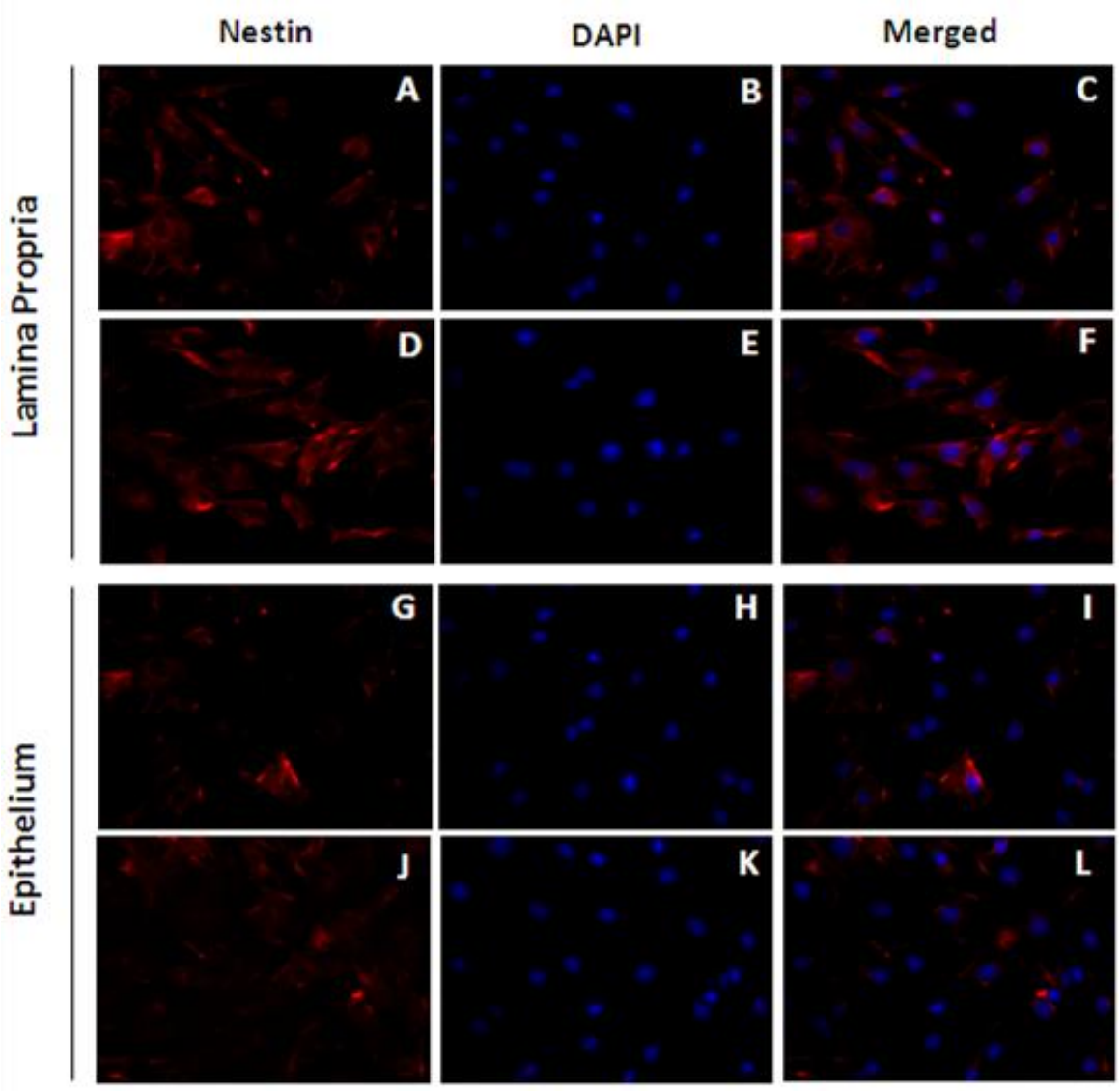

Figure 18 - Nestin immunolocalization in lamina propria (A, D) and epithelium derived stem cells (G, J) isolated from rat 2. Cell nuclei were simultaneously stained with DAPI.

A more detailed view of the nestin expressing cells is presented in Figure 19.

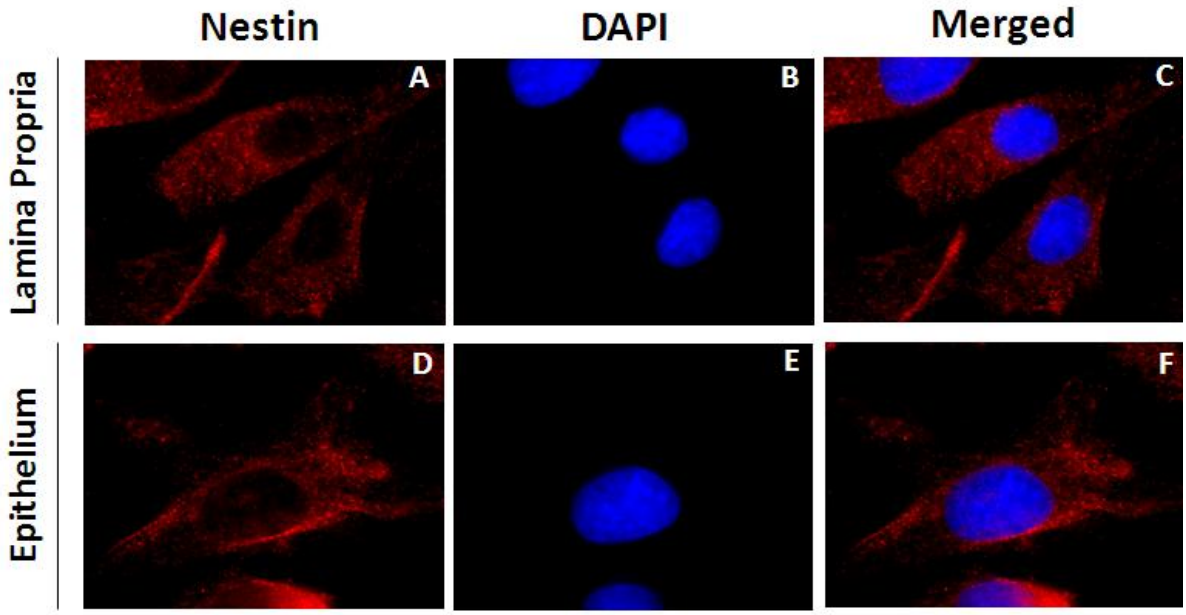

Figure 19 – Nestin immunolocalization in Olfactory stem cells. Cell nuclei were simultaneously stained with DAPI.

4.2 DIFFERENTIATION OF OLFACTORY STEM CELLS INTO OLFACTORY NEUROSPHERES DERIVED CELLS (ONS) AND NEURON LIKE CELL (NLC)

4.2.1 Establishment of the conditions for neurospheres formation

Since we detected that our cultures of both lamina propria and epithelium have olfactory stem cells we carried on with for several experiments.

First of all, conditions for rat olfactory neurospheres formation were examined through three consecutive experiments that were carried out using olfactory derived stem cells isolated from rat 1. To assay the potential of olfactory stem cells for generate neurospheres, they were plated onto poly-D-lysine coated 60 mm cell culture petri dishes filled whit DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml).

As it was reported by Girard et al., 2011; the cells were plated at a density of 16.000 cells per square centimeter. To ensure an accurate estimate of cell number, cell counting was carried out in triplicate using a haemocytometer. For either culture type (lamina propria and epithelium) 4 culture dishes were established and every two days, half of the medium was changed. However, contrary to what has been stated by Girard et al., 2011; olfactory neurospheres were not formed after 2-5 days in culture. Two days after being subjected to neurospheres forming conditions, it is noted that most of the olfactory derived-cells failed to attach to the culture dishes. Comparing to the cells that attached to the culture dishes (elongated and bright cells), cells in suspension were altered in their morphology (round/oval) and color (darker); indicating that they were already dead or dying. At this point, half of the medium was changed in all culture dishes, thereby removing most of these cells.

As seen in Figure 20 (A, C), during approximately 14 days, there were no hints of neurospheres formation either for lamina propria or epithelium derived cultures and the cells remain elongated. The first cell aggregates were visualized in lamina propria (Fig.20; B) and epithelium (Fig.20; D) derived cultures at day 20 and 25. Beyond these cell aggregates (~3 aggregates/dish), a very few number of elongated cells remain dispersed in culture.

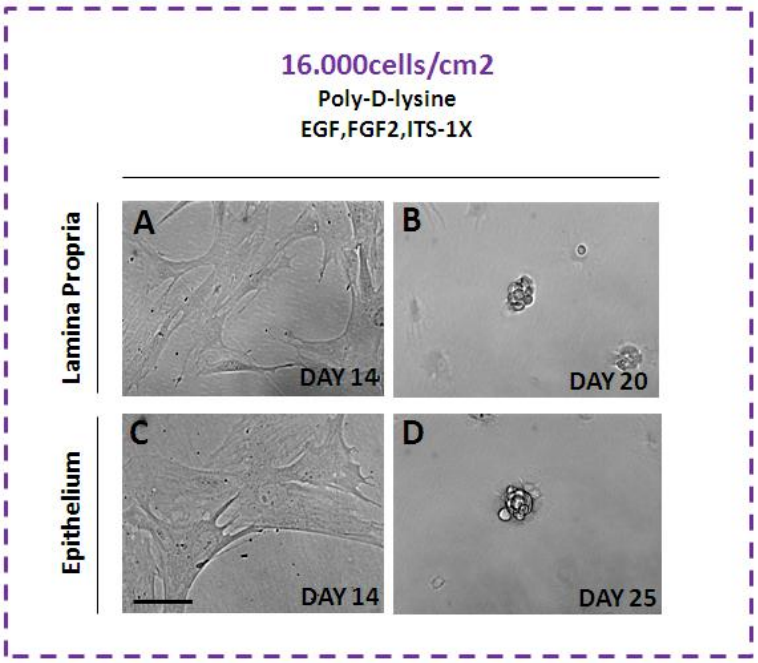

Figure 20 - Phase-contrast microphotographs taken on Lamina propria (A, B) and epithelium (C, D) derived cell cultures. The cells were plated at a density of 16.000 cells per square centimeter on 60mm culture dishes coated with poly-D-lysine and filled with DMEM/HAM F12 supplemented with ITS-X, EGF and FGF2. Scale bar = 100 µm

From the phase-contrast photomicrographs (Fig. 21; A, D) taken on lamina propria and epithelium derived cultures, we could observe that nearly one week after its appearance, the cell aggregates were still the same size (< 100 µm) , indicating a low proliferation rate under neurosphere forming conditions. At that moment, the average number of aggregates reached its peak (~ 5 aggregates/dish); however, a few days later, we could observe that these cell aggregates started to degenerate (Fig. 21; B, C, E, F).

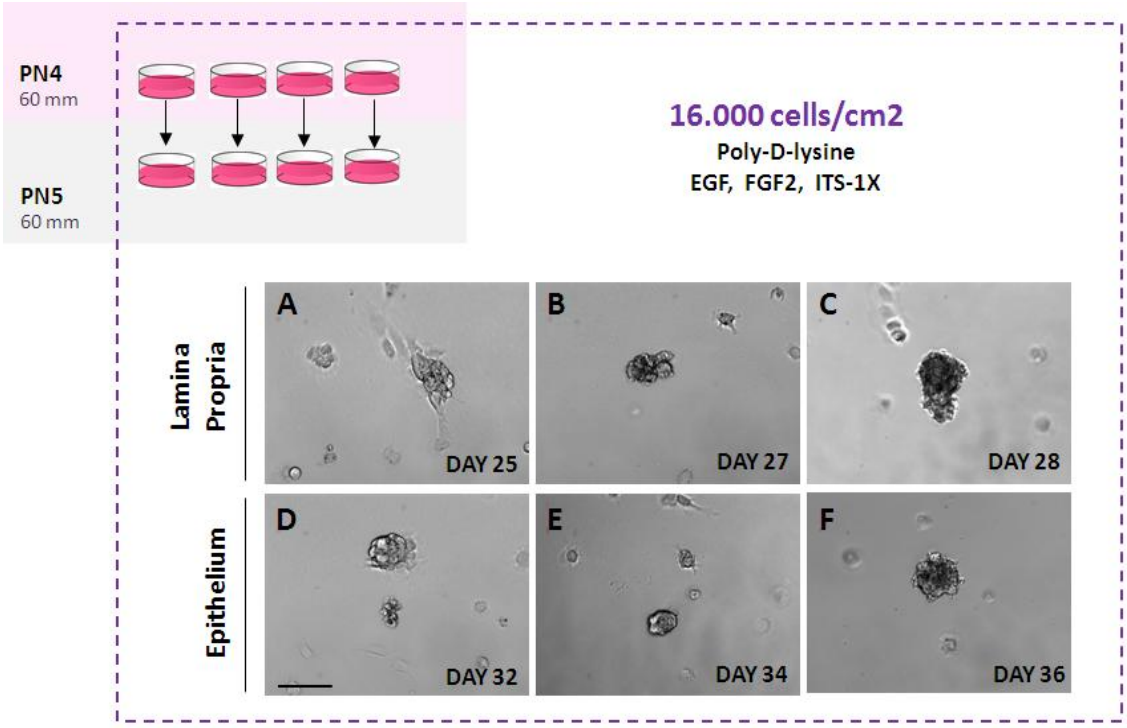

Figure 21 – Formation of olfactory neurospheres from lamina propria and epithelium derived stem cells. The cells were plated in DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml) at a density of 16.000 cells per square centimeter on 60mm culture dishes coated with poly-D-lysine. Phase-contrast microphotographs taken on lamina propria derived cultures at day 25 (A), day 27 (B) and day 28 (C), as well on epithelium derived neurospheres at day 32 (D), day 34 (E) and day 34 (F). PN: passage number; Scale bar = 100µm

As previously described, our experimental procedures were based in a report performed by Girard et. al. which established that 16.000 cells per square centimeter is an optimal plating density to generate olfactory neurospheres. However, using this density, we did not obtained good results.

The initial cell plating density was always decreasing over the time (most of the cells failed to attach to the culture dish after being plated and the few cells that attached to the dish surface failed to proliferate and started to die in culture) and thus, the cells remained scattered throughout the dish surface and failed to produce cells aggregates during approximately 20 days. This observation suggested that was not possible to preserve a favorable environmental niche (i.e.: cell-cell contact) to form olfactory neurospheres.

In order to verify whether the initial cell plating density was too low to allow the formation of neurospheres, we decided to perform a new neurosphere forming assay where the initial cell density was altered and the olfactory neurospheres forming conditions were maintained [poly-D-lysine coated plastic culture dishes (60mm) filled whit DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml)].

The new value of plating cell density was chosen based on previous studies which established that the optimal cell plating density for olfactory neurospheres formation should be 50.000cells/cm^2 (Fig. 22).

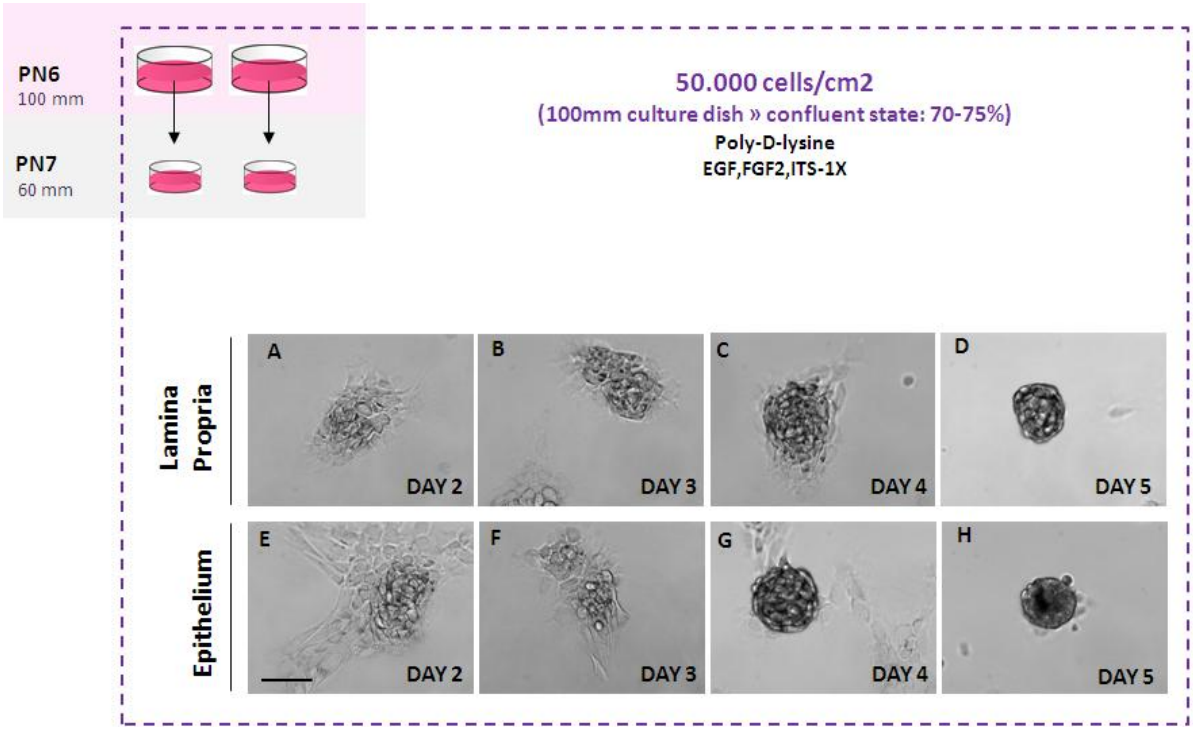

Figure 22 – Formation of olfactory neurospheres from lamina propria and epithelium derived stem cells. The cells were plated in DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml) at a density of 50.000 cells per square centimeter on 60mm culture dishes coated with poly-D-lysine. Phase-contrast photomicrographs taken on lamina propria and epithelium derived cultures at day 2 (A,E), day 3 (B,F), day 4 (C,G) and day 5 (D,H). PN: passage number; Scale bar = 100µm

For either culture type (i.e., lamina propria and epithelium) 2 culture dishes were established and every two days, half of the medium was changed. From the phase-contrast photomicrographs taken on both cultures (Fig. 22 A,E), we could observe that immediately after being plated, olfactory cells displayed a clear tendency to aggregate and form 'islets'. At day 2, most of the cells (~95%) that have been plated under neurospheres forming conditions readily attached to the culture dish and arranged themselves into flat clumps that rapidly proliferate in culture. As seen in Figure 22 (C, G), at day 4 the cells had begun to proliferate at the centre of these flat clumps and had given rise to neurospheres (~ 40 neurospheres/dish), either in lamina propria or epithelium derived cultures. At that moment, the neurospheres were spherical and optically dense with a diameter very similar among them (100-150µm). One day later, (Fig. 22 D,H) about half of the neurospheres floated in the medium while the rest remained attached to the dish surface. Moreover, either free floating or attached neurospheres had a well-defined sphere countour and a diameter slightly smaller compared to the previous day.

Thus, 5 days after being plated, olfactory neurospheres (~ 40 neurospheres/dish) derived from rat 1 were collected for subsequent experiments.

To assess whether neurospheres varied their number and morphological appearance at higher cell plating densities; the entire cell content on a 100mm culture dish in the higher confluent state (95-100%) , was transferred to a 60mm culture dish coated with poly-D-lysine and filled whith DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml) (Fig. 23). For either culture type, 2 dishes were established and every two days, half of the medium was changed.

Comparatively to the previous assay (50.000cells/cm^2 for cell plating density), we observed that the cells tend to form larger aggregates (>100µm) at this higher cell plating density (Fig.23 A, E, B, F); however the de number of aggregates decrease. Furthermore, we detected significant differences in the aggregates morphology.

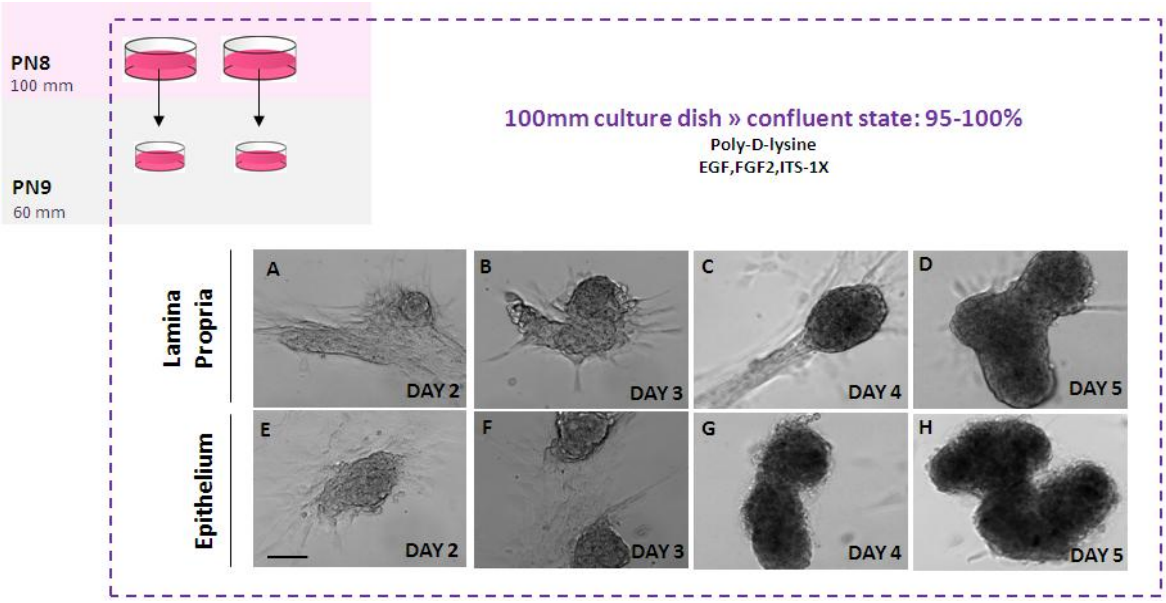

Figure 23 – Formation of olfactory neurospheres from lamina propria and epithelium derived stem cells. After reaching a confluent state of 95-100% on 100mm culture dishes, the entire cell contents in a 100mm culture dish was plated in DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml) on 60mm culture dishes coated with poly-D-lysine. Phase-contrast photomicrographs taken on lamina propria and epithelium derived cultures at day 2 (A,E), day 3 (B,F), day 4 (C,G) and day 5 (D,H). PN: passage number; Scale bar = 100µm

Indeed, the aggregates formed during the last cell plating density assay, were more compact and had and heterogeneous shape; such as arc-shaped (Fig.23 B, D), spoon-shaped (Fig 23. H) or s-shaped (Fig.23 H). Beyond this, there were no spherical aggregates in culture which could be classified as neurospheres. Significant differences between lamina and epithelium derived cultures were not registered once again.

In order to confirm our ability to form neurospheres, we performed additional experiments (rat 2). Olfactory derived cells were plated onto poly-D-lysine coated 60 mm cell culture petri dishes filled whit DMEM/HAM F12 supplemented with ITS-X (1%), EGF (50ng/ml) and FGF2 (50ng/ml). Regarding to their plating density, the cells were plated at 50.000 cells per square centimeter; whereas that appeared to be the most favorable plating density for neurospheres formation (Fig. 24). Cell counting was carried out in triplicate using a haemocytometer and based in all subsequent experiments 5 culture dishes were established for either culture type (i.e.: lamina propria and epithelium).

As seen in Figure 24 (A, B, I, J); 24 hours later, olfactory cells from rat 2 were already attached to the culture dish and organized into clumps that rapidly proliferate either in lamina propria or epithelium derived cultures. Cell aggregates within lamina propria-derived cultures had given rise to neurospheres at day 3 (Fig. 24 E, F); while in epithelium-derived cultures, neurospheres appeared at day 4 (Fig. 24 O, P). For both cultures, the neurospheres were spherical and optically dense with a diameter very similar among them (100-150µm).

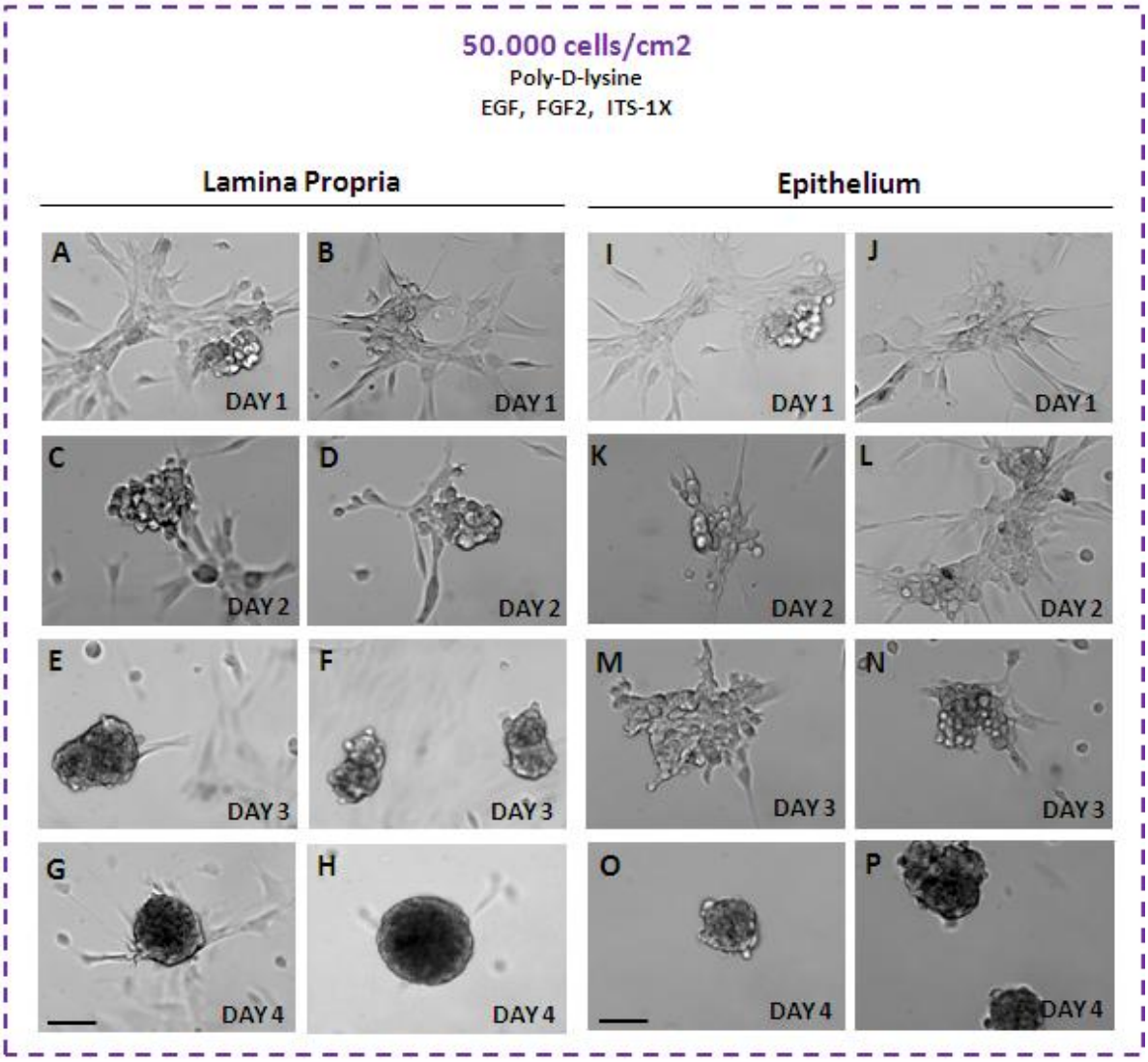

Figure 24 - Phase-contrast photomicrographs taken on lamina propria and epithelium derived cultures at day 1 (A, B, I, J), day 2 (C,D, K, L), day 3 (E, F, M, N) and day 4 (G, H, O, P). PN: passage number; Scale bar = 100µm

At day 5, both in lamina propria and epithelium derived cultures had about half of the neurospheres floating in the medium. At that time, either free-floating or attached neurospheres had a well-defined countour and a darkest tint. Moreover, their diameter appeared to be stable. Thus, 5 days after being plated, olfactory neurospheres (~ 32 neurospheres/dish) derived from rat 2 were also collected for subsquent experiments.

4.2.2 Establishment of the conditions for neurospheres differentiation

As it was previously reported by Girard et al., 2011, olfactory neurospheres-derived cells (ONS) should proliferate as an adherent monolayer from the recently re-plated neurospheres in serum containing culture medium; while the neuron-like cells (NLC) should appear in Neurobasal culture medium after two to three weeks in culture. Thus, in order to assay their ability to differentiate into olfactory neurospheres-derived cells (ONS) and neuron-like cells (NLC), olfactory neurospheres derived from rat 1 were collected and re-plated in serum containing culture medium (DMEDM/ HAM F12 supplemented with 10% FBS and 1% Pen/Strep) and Neurobasal medium containing 1X B-27; 2mM glutamine; 0,025mM glutamate; 1% Pen/Strep and phenol red.

Since some neurospheres were still adherent, we pipette several times up and down to release the maximum number of neurospheres.

For each culture medium, 1 poly-D-lysine coated 60mm cell culture petri dish was established and while the serum containing culture medium was totally renewed every 2 days, half of the Neurobasal medium were renewed every 3 days.

It is important to note that after being mechanically forced to detach from the dish surface, neurospheres remained spherical and compacts as before. Furthermore, they failed to attach to the dish surface either in serum containing or Neurobasal culture medium.

Since the cells failed to attach to the dish surface, in order to preserve them during the medium renewing procedure, both media that we pretended to renew had to be firstly centrifuged. Then, while the supernatant medium was removed the cell pellet was resuspended in fresh culture media and transferred to the same culture dish.

Although this procedure seems to be fairly effective to preserve the floating cells in culture, these cells failed to attach, proliferate and then differentiate into olfactory neurospheres-derived (ONS) and neuron-like cells (NLCs).

From Figure 25, we could observe that at day 15, floating neurospheres had given rise to clumps that was disaggregating in culture and did not seem to proliferate. Moreover, the lamina propria and epithelium derived cells maintained their round shape, either in serum containing (Fig. 25 A, B) and Neurobasal medium (Fig. 25 C, D).

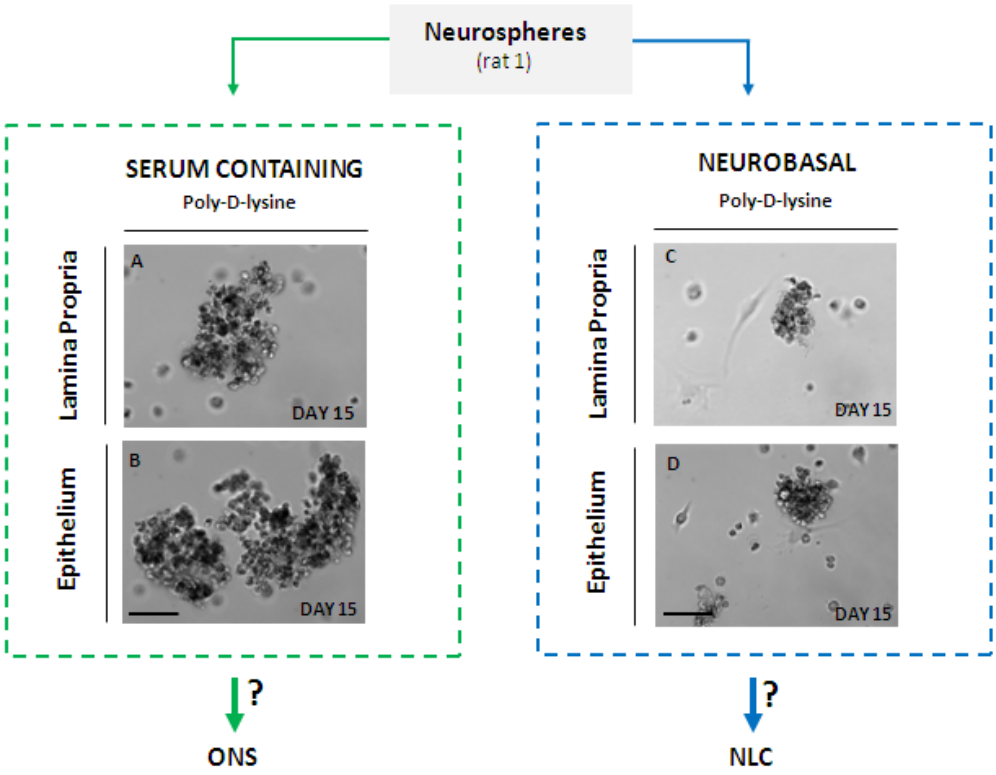

Figure 25 - Phase-contrast photomicrographs taken on lamina propria and epithelium derived neurospheres at day 15. Neurospheres were collected and immediately re-plated in poly-D-lysine coated culture dishes (60mm) filled with serum containing culture medium (*left side*) and Neurobasal medium (*right side*). ONS: olfactory neurospheres-derived cells; NLC: neuron-like cells. Scale bar = 100µm

Approximately 1 month after being re-plated, contrary to what we expected, there were no signs of differentiation. At that time, the cells were still in suspension but they became smaller and darkest as before. Since this clearly indicated that the cells were dying in culture, we decided to discard them.

Once the olfactory neurospheres derived from rat 1 remained spherical and compacts, even after being subjected to several fluxes and refluxes of the culture medium; we decided to assess whether the maintenance of neurospheres tridimensional structure could affect their further differentiation into olfactory neurospheres-derived (ONS) and neuron-like cells (NLC). Thus, neurospheres derived from rat 2 were collected and 'forced' to slightly disaggregate trough trypsin action (1 minute at 37°C) even before being re-plated in serum containing culture medium (DMEDM/ HAM F12 supplemented with 10% FBS and 1% Pen/Strep) and Neurobasal medium containing 1X B-27; 2mM glutamine; 0,025mM glutamate; 1% Pen/Strep and phenol red (Fig. 26).

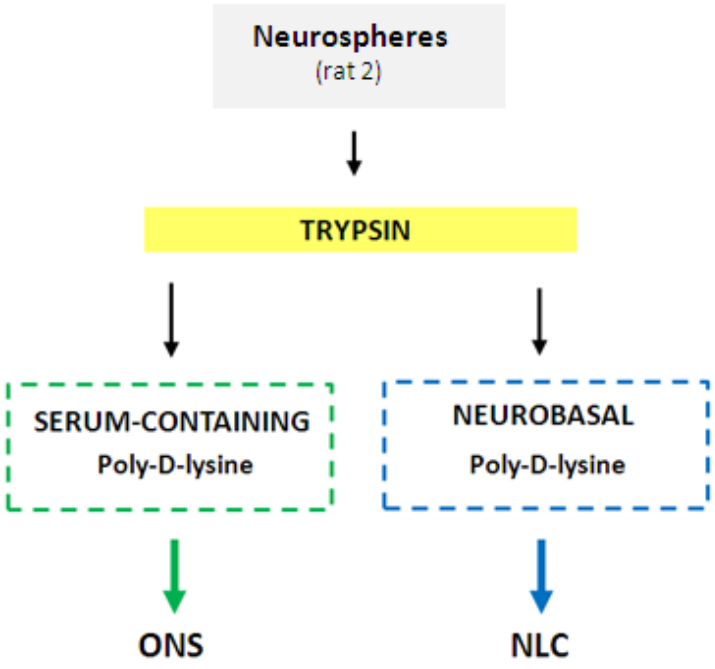

Figure 26 – Outline of the experimental procedure for neurospheres differentiation. ONS: olfactory neurospheres derived cells; NLC: neuron-like cells

Immediately after the trypsin action, there were some single cells in suspension and the neurosphere seemed to be smaller and less compact than before mainly in their periphery. Thus, we have verified that the enzymatic reaction leads to a release of some peripheral cells that were part of the olfactory neurospheres.

4.2.2.1 Neurospheres differentiation into olfactory neurospheres-derived cells (ONS)

We could observe that neurospheres, after being slightly disaggregated trough trypsin action and re-plated in serum containing culture medium, re-attached to the culture dishes and start to differentiate (Fig. 27).

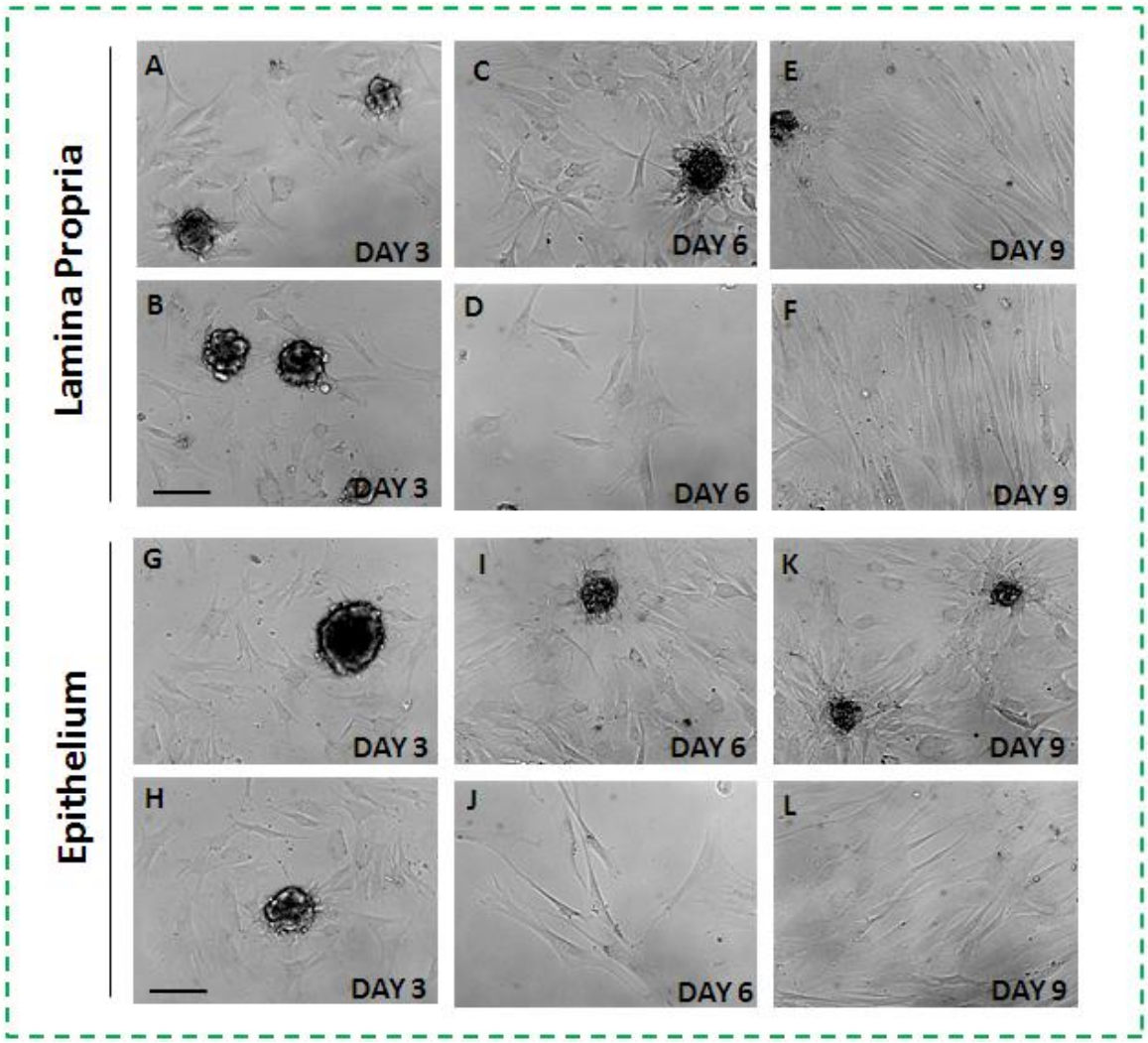

Figure 27 - Phase-contrast photomicrographs taken on olfactory neurospheres-derived cells (ONS) at day 3 (A, B, G, H); day 6 (C, D, I, J) and day 9 (E, F, K, L). Lamina-propria and epithelium derived neurospheres from Rat 2 were slightly disaggregated trough trypsin action (1min at 37^0C) before being re-plated in serum containing culture medium (DMEDM/ HAM F12 supplemented with 10% FBS and 1% Pen/Strep). Scale bar = 100µm

At day 3 (Fig. 27 A, B, G, H); both lamina propria and epithelium derived neurospheres were flattened and dark mainly in the innermost regions and the neurospheres-derived cells (ONS) were proliferating from the neurospheres periphery.

During the next 6 (Fig 27 C-F and I-L) to 8 days, in the same way as the primary cultures, olfactory neurospheres-derived cells (ONS) rapidly proliferated as an adherent monolayer to confluency. Then, these elongated cells were collected and expanded in culture for further experiments. Regarding to their proliferation rate, a doubling time of 48h was registered, and in the course of the time, this value remained relatively constant for both type of cultures (i.e.: lamina propria and epithelium derived ONS).

4.2.2.2 Neurospheres differentiation into neuron-like cells (NLC)

We have also differentiated neurospheres into neuron-like cells (NLC) using Neurobasal medium containing 1X B-27; 2mM glutamine; 0,025mM glutamate; 1% Pen/Strep and phenol red.

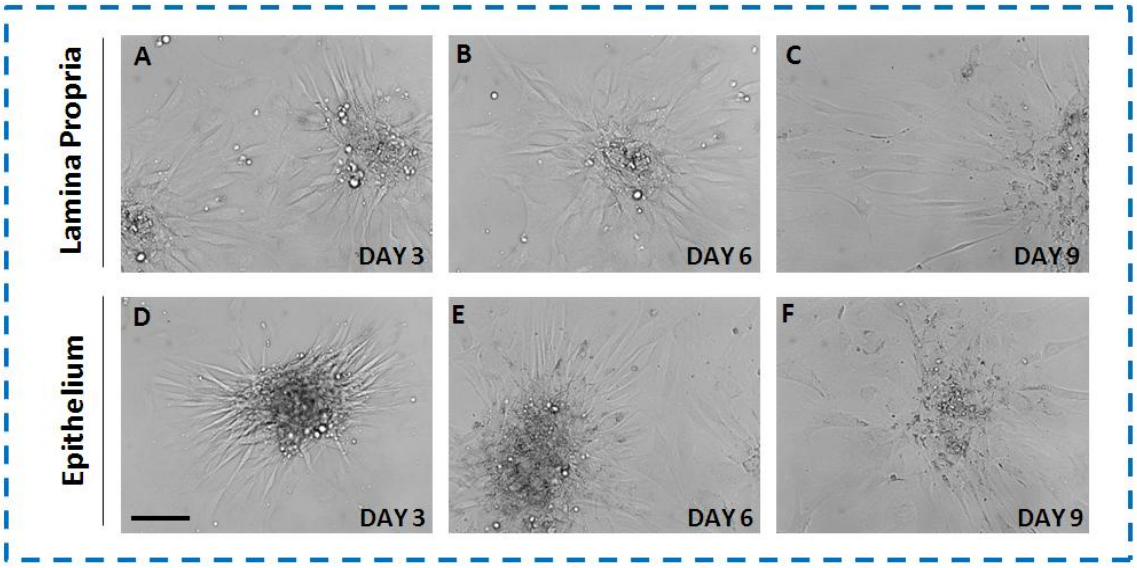

Figure 28 - Phase-contrast photomicrographs taken on olfactory neurospheres-derived cells at day 3 (A, D); day 6 (B, E) and day 9 (C, F). Lamina-propria and epithelium derived neurospheres from Rat 2 were slightly disaggregated trough trypsin action (1min at 37^0C) before being re-plated in Neurobasal medium containing 1X B-27; 2mM glutamine; 0,025mM glutamate; 1% Pen/Strep and phenol red. Scale bar = 100µm

From Figure 28, we could observe that 3 days after being slightly disaggregated trough trypsin action and re-plated in Neurobasal medium, the dissociated neurospheres were completely flattened and their well defined spherical contour was almost imperceptibly. Moreover, we observed that elongated cells radially proliferate from the 'undone' neurospheres. Nevertheless, 3 days later (Fig. 28 B, E) the recently formed cells seemed to be dying and at day 9 (Fig. 28 C, F) the cell death was accentuated.

Since it was previously reported by Girard et al., 2011 that neuron-like cells should appeared in culture after two to three weeks, we decided to maintain these cells in culture. During the third week, we registered an extremely low cellular density, since in the course of time; practically all the cells died in culture and were then discarded during the culture medium renewing procedure.

Nevertheless, as it was previously mentioned by Girard et al., 2011; approximately three weeks after the neurospheres re-plating, we observed cells in culture which resembled neural cells (Fig. 29). However, a few days later, these neuron-like cells died in culture.

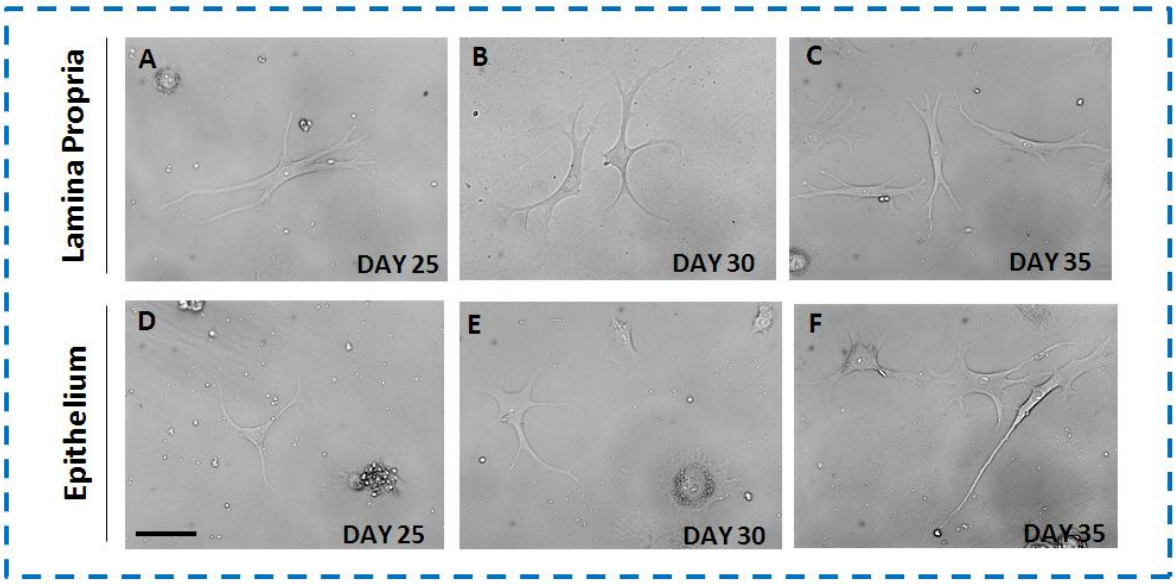

Figure 29 - Phase-contrast photomicrographs taken on olfactory neurospheres-derived cells at day 25 (A, D); day 30 (B, E) and day 35 (C, F). Lamina-propria and epithelium derived neurospheres from Rat 2 were slightly disaggregated trough trypsin action (1min at 37^0C) before being re-plated in serum containing Neurobasal medium containing 1X B-27; 2mM glutamine; 0,025mM glutamate; 1% Pen/Strep and phenol red. Scale bar = 100µm

4.3 CHARACTERIZATION OF OLFACTORY NEUROSPHERS-DERIVED CELLS (ONS) MODEL SYSTEM

Since we successful differentiate olfactory stem cells into olfactory neurospheres-derived cells (ONS) as evidenced by Figure 27; and we would like to use this newly isolated cells as a novel model system for studding neuropathologies, we carried on with several experiments in order to characterize morphologically and biochemically this model.

Morphologically, ONS cells are elongated cells that grow as monolayer of adherent cells (Fig. 30). Regarding to their proliferation rate, a doubling time of 48h was registered, and in a time, this value remained relatively constant for both type of cultures (i.e.: lamina propria and epithelium derived (ONS).

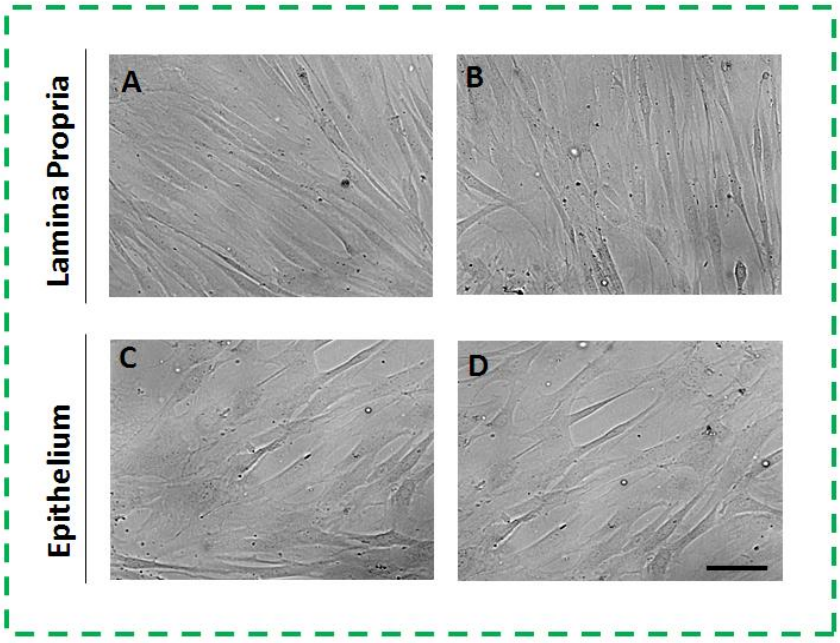

Figure 30 - Phase-contrast photomicrographs taken on olfactory neurospheres-derived cells (ONS) at day 9. Lamina-propria (A and B) and epithelium (C and D) derived neurospheres were slightly disssotiated using trypsin before being re-plated in serum containing culture medium (DMEDM/ HAM F12 supplemented with 10% FBS and 1% Pen/Strep). Scale bar = 100µm

These ONS cells were then expanded by passages and several aliquots were freezed using freezing medium and stored in liquid nitrogen.

In order to confirm whether ONS cells are expressing β-tubulin-III, as previously reported from Girard et al., 2011; lamina propria and epithelium derived ONS cells were immunolabeled with β-tubulin-III monoclonal antibody, while their nuclei were labeled with DAPI. From the immunofluorescence images obtained, we could observe that both lamina propria (Fig. 31 A, C) and epithelium derived ONS cells (Fig. 31 D, F) expressed the neuronal marker β-tubulin-III.

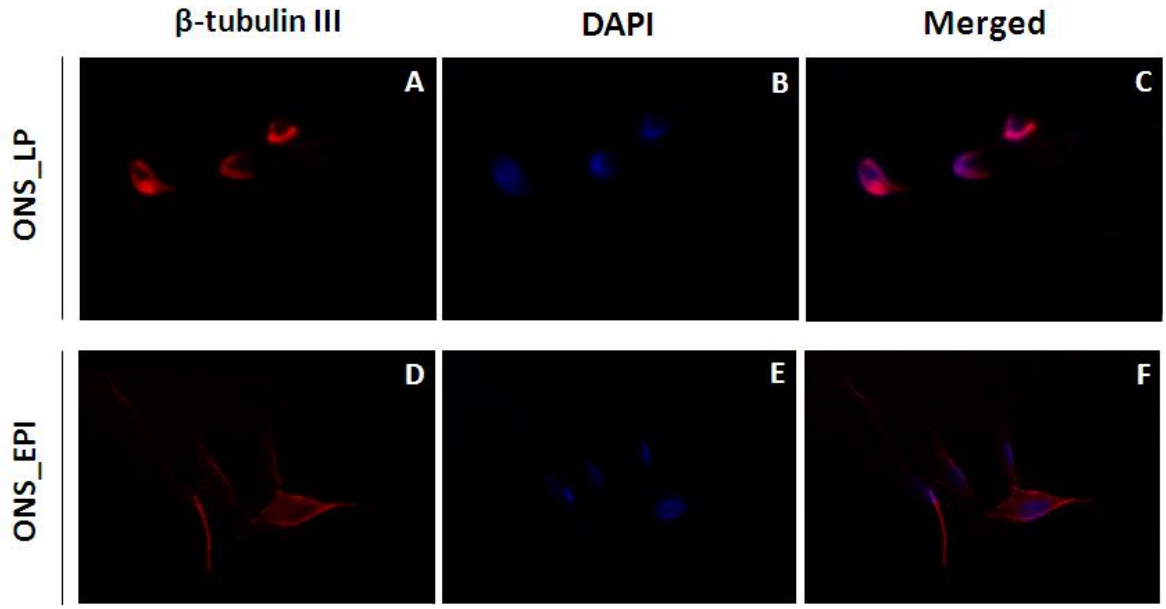

Figure 31 - β-tubulin-III expression in lamina propria (A, C) and epithelium derived ONS cells (D, F). Cell nuclei were simultaneously stained with DAPI (B, E). LP: lamina propria; EPI: Epithelium.

Furthermore, we also performed biochemical analysis of ONS cells that consist in the determination of the expression levels of two relevant proteins for neuropathologies - APP (Amyloid Precursor Protein) and Tau. The expression levels of the stemness marker Nestin were also evaluated. Briefly, we collect cell lysates of isolation/proliferation and differentiation procedures in 1%SDS and after protein content determination they were analyzed by SDS-PAGE followed by immnunoblotting using the specific antibodies for each protein of interest. The results are presented in Figure 32.

In the left panel (Fig. 32), he have the samples collected during the isolation/proliferation and differentiation procedure that consist in nasal mucosa (NM), stem cell of lamina propria (SCLP) and epithelium (SCEPI); neurospheres of lamina propria (NSLP) and epithelium (NSEPI) and the neurospheres-derived cells (ONS) of Lamina Propria (ONSLP) and Epithelium (ONSEPI). In the right panel (Fig. 32) we intent to compare the ONS expression levels of the proteins of interest with other model systems like cell lines (PC12 and SH-SY5Y), rat primary cultures (PCCX) and Rat tissues (rat Cortex, CX and rat hippocampus, H). Regarding APP expressing levels, they were evaluated using the C-Term antibody that recognizes all APP isoforms. The three major APP isoforms, which predominate in most tissues, are APP_{695}, APP_{751} and APP_{770}, both in immature and mature forms. The APP_{695} is referred as neuronal APP isoform (immature form ~ 100 KDa and mature form ~ 130 KDa). Nasal mucosa expressed high levels of immature APP_{695}, while stem cells (SCEPI and SCLP), NS (NSEPI and NSLP) and ONS (ONSEPI and ONSLP) express high levels of $APP_{751/770}$ both in immature (~ 110 KDa) and mature isoforms (~ 140 KDa) (Fig. 32, Panel I).

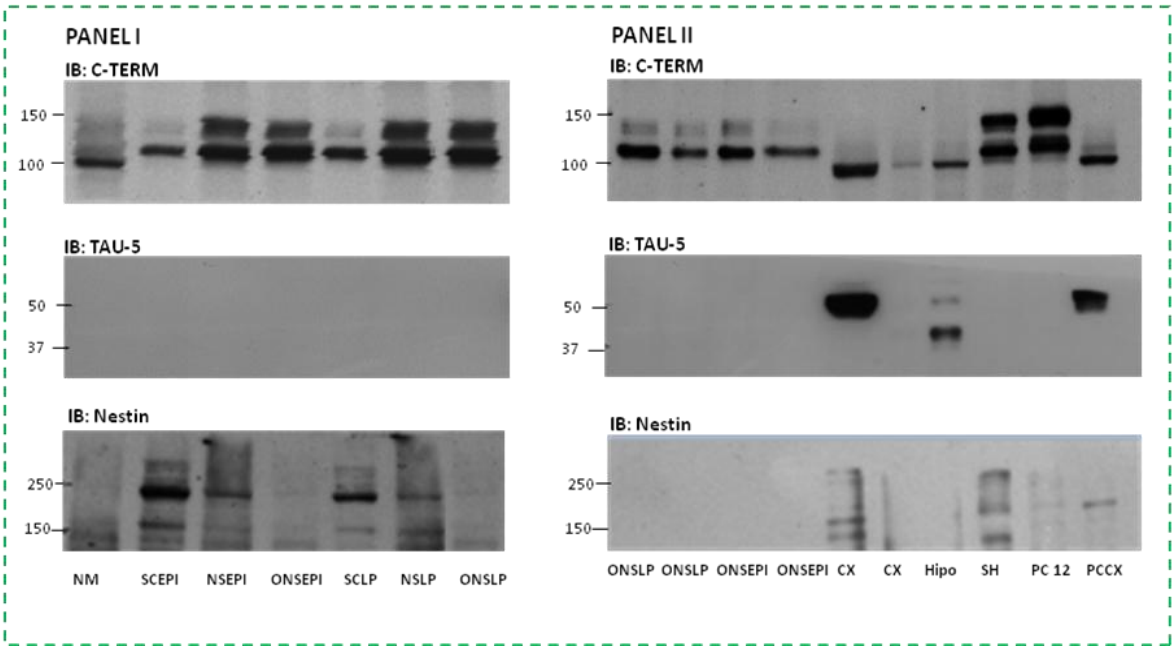

Figure 32 – Comparison of APP, TAU-5 and Nestin expression levels in several samples collected during the isolation/proliferation and differentiation procedure (Panel I) and samples from other model systems (Panel II). Cell lysates were separated by SDS-PAGE followed by immunoblotting (IB) using specific antibodies against APP and Tau isoforms (C-TERM and TAU-5, respectively). An antibody against Nestin was also used. NM, nasal mucosa; SCEPI, Stem cells of epithelium; NSEPI, neurospheres of epithelium; ONSEPI, neurospheres-derived cells differentiated from epithelium (ONSEPI); SCLP, Stem cells of Lamina Propria; NSLP, neurospheres of lamina propria; OLPI, neurospheres-derived cells differentiated from lamina propria; CX, rat cortex; Hipo, Rat hippocampus; SH, SH-SY5Y cell line; PC12, PC12 cell line and rat cortical primary cultures (PCCX).

Curiously, the APP expression pattern is very similar between the ONS and the neuronal-like model systems previously characterized, namely PC12 and SH-SY5Y cell lines

Surprisingly, no Tau is observed in olfactory mucosa derived samples (Fig. 32, Panel I) including in neurospheres-derived cells (ONS). The Tau protein is highly enriched in neuronal systems and therefore abundant in rat cortex and hippocampus samples and also in cortical primary cultures (Fig. 32, Panel II).

Observing the Nestin expression levels in samples collected during the isolation/proliferation and differentiation procedure we clearly realize that the levels are higher in stem cell samples (SCEPI and SCLP) and also in neurospheres samples (NSEPI and NSLP) (Fig. 32, Panel I). In ONS cells the levels of Nestin are very low (Fig. ONS, Panel I) and not detected in Figure 32, Panel II.

5. DISCUSSION

The discovery that neurogenesis continues throughout adult life and the identification of neural stem cells in the adult human brain, has opened exciting new therapeutic options for a wide range of neuropathologies. These cells, allow to overcome most concerns that are usually encountered with other stem cell types, (e.g.: ethical and legal problems related with embryonic stem cells). However, due to their intracranial location, they require a highly invasive surgery for their removal. Moreover, a further understanding of the mechanisms regulating adult neurogenesis under normal and abnormal conditions is still required to the development of novel therapies, either for functional recovery after neurological disorders or trauma (Imayoshi et al., 2011).

More recently, olfactory mucosa being a source of adult stem cells and therefore useful for establishment of model systems to study either the basic mechanisms that contribute to neuropathologies or to develop new diagnostic tools and therapeutic agents; have received much more attention for the scientific community. It has been demonstrated by several studies that new neuronal cells are continuously being generated in this tissue (Duggan and Ngai, 2007; Mackay-Sim, 2010), and contrary to the adult intracranial neuronal stem cells, olfactory mucosa-derived stem cells have the advantageous of being easily and non-invasively harvested in adult humans under local anesthesia (Girard et al., 2011).

Neurogenesis within the olfactory mucosa, as in other renewing tissues, is substantiated by niches of stem cells, located both in the olfactory epithelium and in the underlying olfactory lamina propria (Delorme et al., 2010). Within the olfactory epithelium two distinct populations of stem cells contribute to the neurogenic process throughout life, namely the horizontal basal cells (HBCs) and the globose basal cells (GBCs). However, it has been reported that these epithelial stem cells exhibit a relatively poor proliferation rate (Delorme et al., 2010) and further investigation is required to improve their use either for comparative molecular studies or cell-based therapies (Wetzig et al., 2011). On the other hand, the recently discovered lamina propria-derived stem cells (Tome et al., 2009), have attracted the interest of the researchers which have taken advantage of their easily accessible location, high proliferation rate, ability to proliferate in long-term cultures and tendency to differentiate into neural cells.

After being characterized as members of the mesenchymal stem cells superfamily (Delorme et al., 2010), olfactory ecto-mesenchymal stem cells (OE-MSC) have been emerging as a starting point to establish stem cell based systems to study neuropathologies (i.e.: schizophrenia (Matigian et al., 2010), Parkinson's disease (Matigian et al., 2010; Cook et al., 2011) and familial dysautonomia (Boone et al., 2010)). Additionally and in parallel, it has been reported that OE-MSCs are a promising candidates for stem cell-based therapies; namely after early-onset sensorineural hearing loss (Pandit et al., 2011) or hippocampal lesions (Nivet et al., 2011).

In the work here presented we intended to explore the possibility of establishing in our laboratory an OE-MSCs based model system to study neuropathologies (i.e.: Alzheimer´s disease, Parkinson disease and Dystonia). Based on a very recent study (Girard et al., 2011) we first collected rat olfactory mucosa (O.M) in order to establish primary cell cultures from this tissue. After removing the bone that covered the nasal cavity, the olfactory turbinates (O.T) located above the OM, were easily detected as orange/brown organs (Fig. 13). After incubation in a dispase II solution and under a dissecting microscope with a black background, we could verify that the olfactory lamina propria becomes stripped orange/brown, while the contiguous olfactory epithelium becomes thinner and looks white or translucent (Fig. 14). Based on their color and thickness, these contiguous tissues were separated/isolated and different cultures of each other produced. Nevertheless, and similar to what was previously reported (Murrell et al., 2005; Tome et al., 2009), our experiments indicate that due to the small size of the biological material (approximately 2 mm x 2 mm), the visual barrier between these olfactory tissues is almost imperceptible and thus, the dissection process that should occur along the basement membrane does not guarantee that cells from the other tissue are not present in culture. Taking this into account, and despite the OE-MSCs are derived from olfactory lamina propria; we decided to preserve the putative olfactory epithelium during the dissection procedure and use both tissues for the further procedures.

In general, from the results obtained we can verify that only slight differences between epithelium and lamina propria derived cultures are observed despite their identical primary cultures morphological appearance (Fig. 16), expression of the stemness

marker nestin (Fig. 17; Fig. 18 and Fig.19) or ability to form neurospheres (Fig. 22 and Fig.24) which can subsequently proliferate as olfactory neurospheres-derived cells (Fig. 27) or terminally differentiate into neuron-like cells (Fig. 28 and Fig. 29). Moreover, there were no significant differences in the neurospheres morphology, size or number.

Since it has been reported that the stemness marker nestin is expressed both by epithelium and lamina propria-derived stem cells and these two distinct cell types displaying the same ability to form neurospheres (Tome et al., 2009); to establish a stem cell based model system to study neuropathologies in our laboratory, there is an additional need of examining the expression of specific cell markers both for olfactory lamina propria and epithelium derived stem cells. Based on a previous study (Tome et al., 2009) it is reasonable to deduce that the future work to determine which types of cells are growing in culture will be to assess the expression of two tissue-specific cell markers, namely Stro-1 and Cytokeratins. While the mesenchymal stem cell marker Stro-1 was expressed by lamina propria-derived cells but not by the cells of the olfactory epithelium; Cytokeratins (broad spectrum antibody) were expressed by two types of epithelial cells (i.e.: horizontal basal cells and sustentacular cells) but not by lamina propria-derived cells (Tome et al., 2009).

Once established that primary cultures isolated from rat olfactory mucosa were proliferating as adherent monolayers of elongated cells according with previously described, we subsequently evaluated the expression of the stemness marker nestin (Fig. 17; Fig. 18 and Fig 19). The assessment of nestin expression in both cultures revealed that we successfully isolated olfactory stem cells and then, several experiments were carried out using these olfactory stem cells.

First of all, to assay the potential of olfactory stem cells for generate neurospheres, olfactory cells from rat 1 were plated at a density of 16.000 cells per square centimeter on poly-D-lysine coated cell culture petri dishes filled with DMEM/HAM-F12 supplemented with ITS, EGF and FGF2 (Fig. 20 and Fig. 21). As already mentioned, these experimental conditions to form olfactory neurospheres were previously described by Girard et al.

Nevertheless, contrary to what has been stated in that study; after 2-5 days there were no neurospheres in our cultures and even after 2 weeks, there were no hints of neurospheres formation (Fig. 20; A, C). After 3 weeks under neurospheres forming conditions, small cell aggregates appeared both in lamina propria (Fig. 20; B) and epithelium derived cultures (Fig. 20; D); however a few days later we observed that these cell aggregates were degenerating and their cells were dying in culture (Fig. 21).

Since the study from Girard et al. is focused exclusively on lamina propria derived olfactory ecto-mesenchymal stem cells (OE-MSCs), one hypothesis arises to explain the failure in the neurospheres formation within lamina propria derived cultures: as we suspected, the dissection method to separate the tissues within the olfactory mucosa does not guarantee that epithelial cells are not present in lamina propria derived culture. This means that the number of OE-MSCs that were available to form neurospheres would be less than 16.000cells/cm^2 and thus, the minimum density to generate neurospheres is not assured.

In order to verify whether the initial cell plating density was too low to allow the formation of neurospheres, we decided to perform a new neurosphere forming assay. The new value for cell plating density was chosen based on previous studies (Wetzig, 2006; Wetzig et al., 2011) which established that the optimal cell plating density for olfactory neurospheres formation should be 50.000cells/cm^2. In this context, it is important to note that in these studies, to establish primary cultures from rat olfactory mucosa, the lamina propria-derived cells and olfactory epithelium derived cells were purposely combined before being plated in serum-containing culture medium. By analyzing the phase contrast photomicrographs taken on both cultures during this neurospheres forming assay (Fig. 22), it is evident that after being plated at a density of 50.000cells/cm^2, almost all cells (~95%) attached to the culture dish and arranged themselves into flat clumps that rapidly proliferate in culture (Fig. 22; A,E). After 4 days under neurospheres forming conditions the cells had given rise to neurospheres (~40neurospheres/dish), either in lamina propria or epithelium derived culture (Fig. 22; C,G). To assess their ability to differentiate into olfactory neurospheres-derived cells (ONS) and neuron-like cells (NLCs); at day 5 (Fig.22; D, H) olfactory neurospheres derived

from rat 1 were collected and re-plated either in serum-containing culture medium or Neurobasal medium; as will be discussed below.

During the last assay to determine the most favorable density to form neurospheres from our primary cultures, olfactory cells were plated at higher cell plating densities (Fig. 23) under the same neurospheres forming conditions. Our experiment revealed that the cells did not form neurospheres; which means that too many cells in culture also may create a niche that inhibits neurospheres formation. In order to confirm that primary stem cell cultures are able to generate neurospheres, olfactory cells previously isolated from rat 2 were also plated under neurospheres forming conditions at a density of 50.000 cells/cm^2; whereas that appeared to be the most favorable plating density for neurospheres formation. As shown in Figure 24, this density proved suitable for this neurospheres forming assay; once after 4 days under neurospheres forming conditions the cells had given rise to neurospheres either in the lamina propria or epithelium derived cultures.

Then, like olfactory neurospheres from rat 1, olfactory neurospheres from rat 2 were collected and re-plated either in serum-containing culture medium or Neurobasal medium; also to assess their ability to differentiate into olfactory neurospheres derived cells (ONS) and neuron-like cells (NLCs). As previously reported by Girard et al., olfactory neurospheres-derived cells (ONS), should proliferate as an adherent monolayer from the recently re-plated neurospheres in serum containing culture medium; while the neuron-like cells (NLC) should appear in Neurobasal culture medium after two to three weeks in culture. In the first experiment, which was carried out on olfactory neurospheres from rat 1; the neurospheres remained spherical and compacts even after being forced to detach from the dish surface with medium fluxes and refluxes, and perhaps because of this they failed to re-attach, proliferate and subsequently differentiate either in ONS or NLCs (Fig. 25). Thus, and since the results obtained seemed to indicate that the maintenance of the neurospheres tridimensional structure may prevent the attachment of the neurospheres to the dish surface both in serum-containing and Neurobasal medium; olfactory neurospheres derived from rat 2 were collected and ‘forced’ to slightly disaggregate trough trypsin action and then re-plated in both media.

As shown in Figure 27, after being re-plated in serum-containing culture medium, these neurospheres successfully re-attached to the new dish surface and from the third day, olfactory neurospheres-derived cells (ONS) rapidly proliferated as an adherent monolayer from the neurospheres periphery. We have also differentiated neurospheres into neuron-like cells (NLC) in Neurobasal medium (Fig. 28 and Fig. 29). Nevertheless, we registered an extremely low cell density, since in the course of time; practically all the cells died in culture, suggesting that additional experiments should be performed in order to optimize the differentiation conditions (e.g: adding nerve growth factor - NGF - to the culture medium)

Characterization of the ONS model system was also performed. The preliminary morphological and biochemical characterization of the ONS models system was achieved and revealed that our ONS model system in term of APP and Tau expression levels behaves similarly to neuronal-like model systems previously characterized including PC12 and SH-SY5Y cell lines. However, additional characterization should also be performed.

In conclusion, our results strength the hypothesis of using stem cell based model systems to study the cellular and molecular mechanisms underlying several neuropathologies, including Alzheimer's disease.

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