Universidade de Aveiro Secção Autónoma de Ciências da Saúde
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Sónia Raquel Capela Sacramento

Sistemas modelo baseados em células estaminais da mucosa olfactiva

Establishing model systems from olfactory mucosa stem cells

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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 Sandra Maria Tavares da Costa Rebelo, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro, e sob a co-orientação 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.

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Dedico este trabalho aos meus avós, Carlos e Etelvina.

o júri

presidente

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palavras-chave

Células estaminais, Mucosa olfactiva, Neurosferas, Células derivadas de neurosferas, Células tipo neuronais

resumo

As células estaminais são uma classe distinta de células, devido às suas capacidades de regeneração e diferenciação em vários tipos de células especializadas. Os nichos onde estas se encontram servem-lhes de sustentação e permitem a sua manutenção num estado indiferenciado, afectando a sua regeneração e diferenciação através de estímulos. As células estaminais dividem-se em duas classes: embrionárias (pluripotentes) e adultas (multipotentes), embora também existam células estaminais pluripotentes induzidas.

Nos últimos anos tem-se estudado a possibilidade de utilizar sistemas baseados em células estaminais no estudo de neuropatologias. Assim sendo, os objectivos desta dissertação foram o isolamento e proliferação de células estaminais da mucosa olfactiva, a indução destas células para a formação de neurosferas e a sua diferenciação para células tipo neuronais (NLC) e células derivadas de neurosferas (ONS). Também se procedeu à diferenciação das ONS e à caracterização dos modelos celulares NLC e ONS. Para se atingir os objectivos definidos, foram recolhidas biópsias de mucosa olfactiva e isolaram-se células estaminais de epitélio e da lâmina própria. As células estaminais da mucosa olfactiva proliferaram e foram induzidas a formar neurosferas com um meio de cultura específico (DMEM/F12 com ITS-X, EGF e FGF2). As neurosferas foram posteriormente diferenciadas em células ONS (com meio DMEM/F12) e em NLC (com meio neurobasal suplementado com NGF, B27, glutamina e glutamato). Imagens obtidas durante o tempo de diferenciação das NLC foram analisadas tendo em conta parâmetros morfométricos. As células ONS foram adaptadas à cultura em meio sem soro e diferenciadas em células tipo neurónios (usando meio DMEM/F12 com N2 e meio DMEM/F12 com B27).

Os resultados obtidos indicam que estabelecemos culturas primárias de células estaminais da mucosa olfactiva de rato. A eficiência dos protocolos de isolamento e proliferação foi confirmada pela marcação com nestina através de imunofluorescência e pela formação de neurosferas. A análise morfométrica das NLC indicou que diferenciámos as neurosferas para células tipo neuronal, devido à sua morfologia neuronal e à expressão do marcador neuronal β-tubulina III. Foram também estabelecidas culturas de células ONS, posteriormente diferenciadas através da redução de soro, apresentando um fenótipo tipo neuronal quando mantidas em meio definido. Contudo, devem ser realizadas experiências futuras para a caracterização deste novo modelo celular. Os nossos resultados permitem-nos concluir que estabelecemos e caracterizámos novos sistemas modelo baseados em células estaminais. Estes resultados são relevantes uma vez que tais modelos podem ser usados para o estudo de mecanismos celulares e moleculares envolvidos em inúmeras neuropatologias, nomeadamente na Doença de Alzheimer.

keywords

Stem cells, Olfactory mucosa, Neurospheres, Olfactory neurosphere-derived cells, Neuron-like cells

abstract

Stem cells are a distinct class of cells, characterized by their ability to self-renew and differentiate into several specialized cell types. The niche of stem cells provides them support, favors their existence in an undifferentiated state and affects, by stimuli, their self-renewal and cellular fate. Stem cells can be divided in two broad classes: embryonic (pluripotent) and somatic stem cells (multipotent), although induced pluripotent stem cells are also a reality nowadays.

The possibility of investigating neuropathologies using stem cell based systems has attracted interest among researchers in the last few years. Therefore, the main objectives of this dissertation were the isolation and proliferation of olfactory mucosa stem cells that were further induced to form neurospheres and further differentiated into neuron-like cells (NLC) and olfactory neurosphere-derived cells (ONS). ONS differentiation and the characterization of NLC and ONS model systems were also performed. For the accomplishment of these objectives, olfactory mucosa biopsies were collected and epithelium and lamina propria stem cells isolated. The well proliferating olfactory mucosa stem cells were induced to form neurospheres using a specific culture medium (DMEM/F12 supplemented with ITS-X, EGF and FGF2). The neurospheres were then differentiated into ONS cells (using DMEM/F12 medium) and into NLC (using neurobasal medium supplemented with NGF, B27, glutamine and glutamate). Morphometric analysis of neuron-like cells was performed on microphotographs taken at several time points during the differentiation procedure. ONS cells were adapted to serum deprivation and differentiated into neuronal-like cells (using DMEM/F12 with N2 medium and DMEM/F12 with B27 medium).

Our results indicate that we successfully established primary rat cultures from olfactory mucosa stem cells. The efficiency of the isolation/proliferation procedure was confirmed by positive immunostaining with stemness marker nestin and also by their ability to form neurospheres. The morphometric analysis of NLC revealed that we successfully differentiated neurosphere-forming cells into neuron-like cells, since they assume a neuronal like phenotype and they highly express the neuronal marker β-tubulin III. Additionally, ONS cultures were established and further differentiated by gradual serum deprivation. In fact, these cells presented neuronal-like phenotypic characteristics when cultured in defined medium. However, additional experiments for characterization of this new model system should be performed. From our results we can conclude that we efficiently established and characterized new stem cells model systems. These results are of paramount importance since they will be used for the study of cellular and molecular mechanisms underlying several neuropathologies, including Alzheimer's disease.

INDEX

ABBREVIATIONS

AOB	Accessory olfactory bulb
AON	Anterior olfactory nuclei
BL	Basal lamina
BM-MSCs	Bone-marrow mesenchymal stem cells
BSA	Bovine serum albumine
CNS	Central nervous system
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EC	Entorhinal cortex
ECM	Extracelular matrix
EDTA	Ethylenediamine tetraacetic acid
EGCs	Embryonic germ cells
EGF	Epidermal growth factor
ENT	Ear nose and troat
epiSCs	Epiblast stem cells
ESCs	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
GBCs	Globose basal cells
GFAP	Glial fibrillary acid protein
HBCs	Horizontal basal cells
HSCs	Hematopoietic stem cells
H	Hypothalamus
ICM	Inner cell mass
iPSs	induced pluripotent stem cells
ITS-X	Insulin, Transferrin, Selenium
LA	Lateral amygdala
LP	Lamina propria
MAB	Monoclonal antibody

MAP2	Microtubule-associated protein
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
MSC	Mesenchymal stem cell
NGF	Nerve growth factor
NLC	Neuron-like cells
OE	Olfactory epithelium
OECs	Olfactory ensheathing cells
OE-MSCs	Olfactory ecto-mesenchymal stem cells
OM	Olfactory mucosa
ONS	Olfactory neurosphere-derived
ORN	Olfactory receptor neurons
OSN	Olfactory sensory neuron
OT	Olfactory tubercule
PBS	Phosphate Buffered Saline
PC	Piriform cortex
Pen/Strep	Penicilin/Streptomycin
PN	Passage number
RMS	Pstral migratory stream
ROS	Reactive oxygen species
SGZ	Subgranular zone
SMA	Smooth muscle actin
SUS	Sustentacular cells
SVZ	Subventricular zone
TDC	Terminally differentiated cell
US	United States
VA	Vomeronasal amygdala
VNO	Vomeronasal organ

1. INTRODUCTION

1.1. Stem cells

The human body is composed by over two hundred different cell types that are organized into tissues and organs, providing all the functions required for its viability and reproduction (1). The cellular developmental potency (range of commitment options available to a cell) is progressively narrowed as development proceeds from a fertilized egg to the adult. Zygotes and early blastomers are thought to be the pinnacle of the cellular hierarchy of developmental potency, due to their totipotency (ability to orchestrate the formation of an entire organism, including extra-embryonic tissues such as placenta). However, unlike stem cells, fertilized eggs are not able to self-renew by simple cell division (2, 3).

Stem cells are a distinct class of cells, since they preserve, to varying extents, the potential for multi-lineage differentiation (2, 4). Thereafter, these cells are defined by their self-renewal and differentiation ability, as they are able to unlimitedly produce daughter cells equivalents to themselves but with more restricted properties (Figure 1) (5, 6).

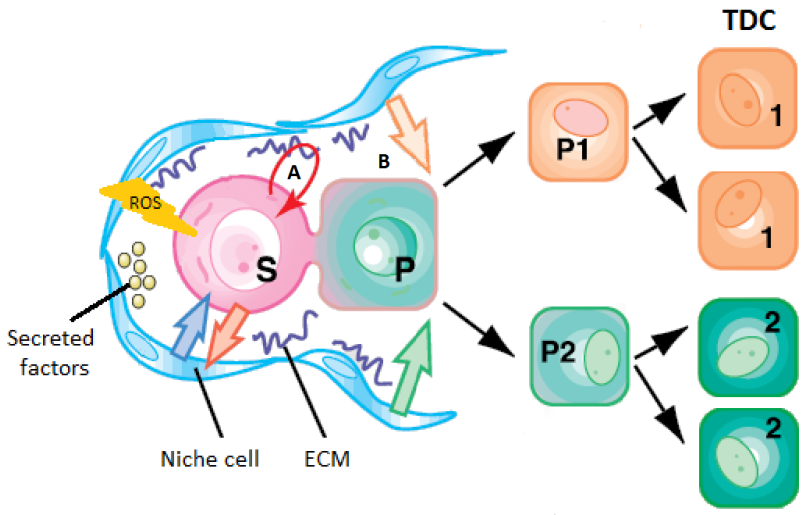

Figure 1 - Differentiation of stem cells. Stem cells behavior is influenced by its niche, formed by the stem cell (S) and niche cells, and extracellular matrix (ECM) proteins, secreted factors and physical factors, such as stiffness and stretch (blue and red thick arrows). This environment allows stem cells to divide asymmetrically to produce (A) a stem cell that remains in the niche and (B) a progenitor cell, that depending on the combination of extrinsic factors to which they are exposed can differentiate along different pathways (1 and 2) to generate distinct terminally differentiated cells (TDC). ROS – reactive oxygen species. P – progenitor cell, P1 – progenitor cell 1, P2 – progenitor cell 2. Adapted from (7).

Mammalian stem cells can divide in a symmetric or asymmetric manner in order to regulate their number and tissue homeostasis. The symmetric division enlarges the stem cells pool while asymmetric cell division generates one daughter cell that remains at the niche and one more rapidly-dividing cell that exits and differentiates and is named progenitor cell (Figure 1) (8-

11). Also known as transient amplifying cells, these committed mature progenitors with limited proliferative capacity and restricted differentiation potential are responsible for increasing the number of differentiated cells produced by each stem cell division (Figure 1) (7, 10).

The niche of stem cells is the cellular microenvironment that provides them support, favors their existence in an undifferentiated state and affects, by stimuli, self-renewal and cellular fate (4). Aspects such as adhesion to extracellular matrix (ECM) proteins, direct contact with neighboring cells, exposure to secreted factors and physical factors like oxygen tension and sheer stress are known to influence stem cells behavior (Figure 1) (1).

Stem cells can be divided in two broad classes according to their origin, that are different in the degree of developmental potency: embryonic and somatic stem cells, although induced pluripotent stem cells are also a reality nowadays (Figure 2) (12).

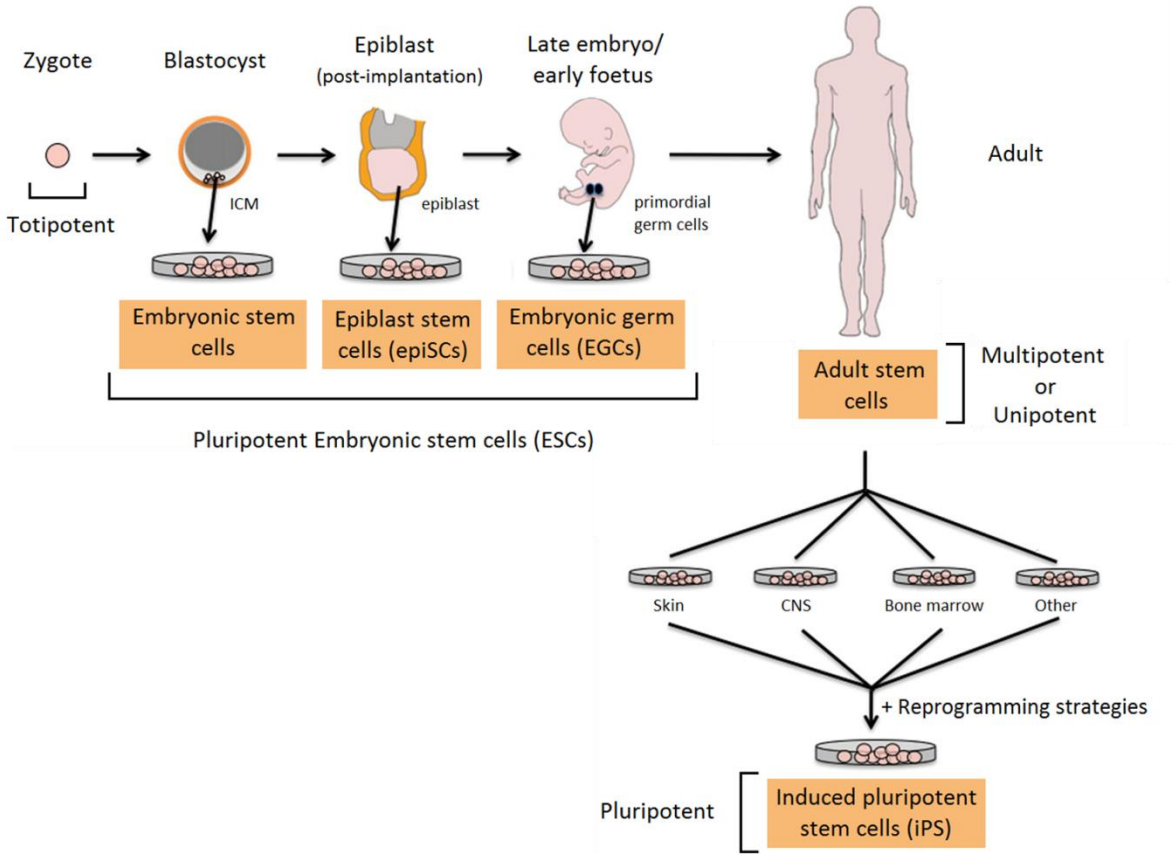

Figure 2 - Types and origin of stem cells. Cells are described as totipotent if they can form an entire organism, including extraembryonic tissues. If they can form all cell types of the adult organism they are named pluripotent. Embryonic stem cells can be derived from inner cell mass (ICM) of blastocysts, post-implantation epiblast and primordial germ cells. In adults, there can be found multipotent and unipotent cells (only one differentiated lineage), that can be reprogrammed to plutipotency by several reprogramming strategies. epiSCs – epiblast stem cells, EGCs – embryonic germ cells, ESCs – embryonic stem cells, CNS - central nervous system, iPS – induced pluripotent stem cells. Adapted from (1).

1.1.1. Embryonic stem cells

Embryonic stem cells (ES cells) are the most potent, pluripotent stem-cell lines derived from the inner cell mass of pre-implantation mammalian embryos or the early blastocyst, before formation of the tissue germ layers (4). Moreover, ES cells can also be derived from primordial germ cells (EGCs) that arise in the late embryonic and early fetal stage of development (13). These cells have two distinct properties: unlimited self-renewal capacity and pluripotent developmental potency similar to their embryonic founder cells, being able to differentiate into cells and tissues of all three germ layers (ectoderm, mesoderm and endoderm) *in vivo* and *in vitro* (2, 5).

ES cells were firstly isolated in 1981 from mouse blastocyst (14) and were first derived from human blastocysts in 1998 (15). Ten years later, in January 2009, the US Food and Drug Administration approved the first clinical trial involving human ES cells, to evaluate the safety of ES cell-derived oligodendrocytes in repair of spinal cord injuries (1). Nowadays, several human ES cell lines exist and banking of clinical grade cells is ongoing.

These cells are particularly interesting for researchers since they allow the analysis of the relationships between gene function and cell and tissue development, as well as explore early human development through *in vitro* differentiation, which recapitulates aspects of normal gastrulation and tissue formation (15, 16).

In spite of the actual ethical issues and challenges on defining specific cell types and routes of transplantation and engraftment, ES cells represent a very attractive model since they could also be a source of cells for cell-based therapy with optimal immunological matching of donor and recipients (1, 2).

1.1.2. Somatic stem cells

Somatic stem cells, also known as postnatal or adult stem cells (although they can be extracted from newborns and adolescents), are undifferentiated cells found among differentiated cells within a tissue or organ. They can actively replenish themselves through self-renewal and regenerate the multiple lineages that comprise an entire tissue or tissues, therefore these cells are considered multipotent. The pools of somatic stem cells are responsible for the regeneration of highly proliferative tissues (that turn over rapidly throughout adulthood, replacing their mass in a matter of days), such as blood, skin and gut epithelia (2, 4). Nevertheless, stem cells are also present in tissues that normally undergo very limited regeneration, such as the brain and liver.

Adult stem cells are maintained in a quiescent state (G0 state, out of cell cycle and in low metabolic state), though they are able to exit quiescence and rapidly expand and differentiate in response to an external stimuli like the stress (17). However, for many adult stem cell types, there are problems with accessibility, low frequency (<1/2% of the total cellularity), restricted differentiation potential and poor *in vitro* growth (5, 18).

Recent work has questioned the lineage restricted characteristics of adult stem cells, by the observation that some multipotent cells can transdifferentiate when relocated, originating other specialized cells appropriate to their new niche (18-20). Transdifferentiation means that somatic multipotent stem cells of tissues such as the hematopoietic system, the intestine or the skin are inherently plastic and capable of generating cell types outside of their primary lineage (e.g. hematopoietic stem cells contributing to non-hematopoietic tissues). However, transdifferentiation concept is currently a controversial issue, only rarely observed (3, 21).

According to their own features, somatic stem cells can be divided into several groups, from which hematopoietic, mesenchymal, epithelial and neural stem cells are the best well studied and characterized.

1.1.2.1. Neural stem cells

Neogenesis of mature cells persists throughout adult life within discrete brain regions of the central nervous systems (CNS) of all mammals (22). In humans, it occurs predominantly in the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and in the subventricular zone (SVZ) of the forebrain lateral ventricles (23). This process is crucial for the maintenance of brain integrity, plasticity and optimal function, due to its central role in the generation and integration, both functional and synaptic, of new neurons into pre-existing neural networks.

During the differentiation process in SVZ, multipotent type B astrocytes (stem cell), that were identified as the bona fide SVZ stem cells, give rise to fast-cycling transiently proliferating precursor cells that are called type C precursors (transiently amplifying cell). These precursors generate mitotically active type-A neuroblasts that, while dividing, migrate tangentially towards the olfactory bulb, where they move to the outer cell layers (Figure 3). Once there, they integrate as new interneurons - periglomerular and granule neurons (22, 23).

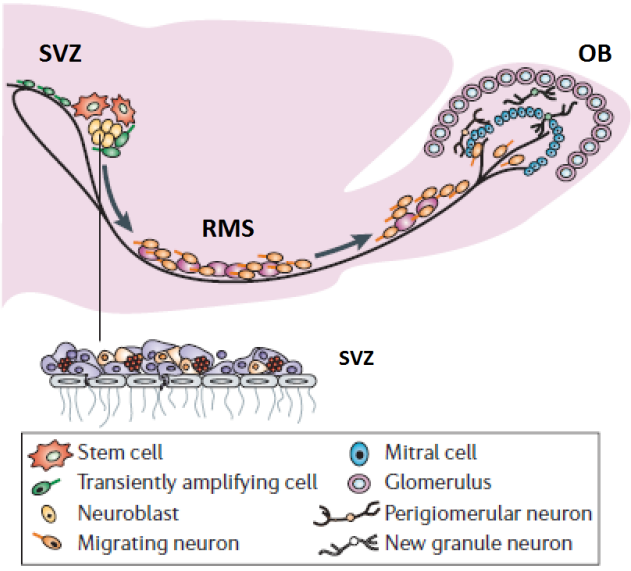

Figure 3 - Generation of new interneurons in the subventricular zone (SVZ). Sagittal section through the lateral ventricle, showing the larger area of adult neurogenesis. **The type B astrocytes** (red) give rise to type C precursors (green) and, sequentially, to type A neuroblasts (yellow) that migrates through the rostral migratory stream (RMS) till the olfactory bulb (OB). Adapted from (23).

In the SGZ is seen a slightly similar cellular hierarchy, with the type B astrocyte as the true stem cell. These astrocytes produce the intermediate type D precursors that eventually give rise to the type G granule neurons, that will integrate functionally into the granule cell layer (22, 23) (Figure 4).

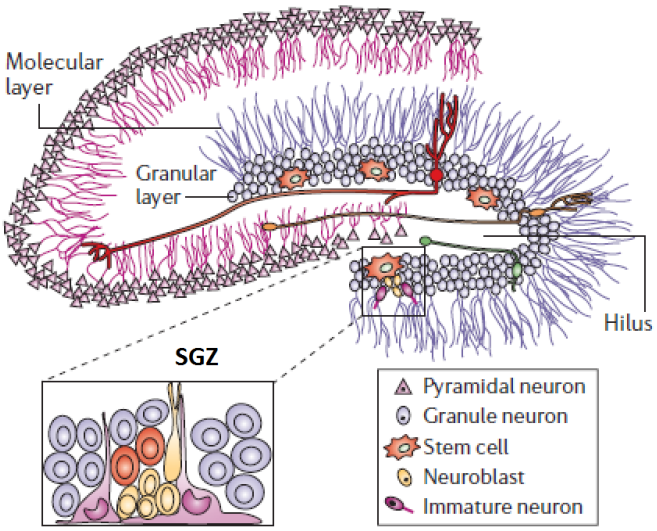

Figure 4 - Generation of new granule neurons in the subgranular zone (SGZ). Schematic representation of the dentate gyrus and the subgranular zone. Type B astrocytes (red) give rise to type D precursors (green) and then to type G granule neurons (blue), that will integrate into the granule cell layer. Adapted from (23).

Adult neural stem cells were firstly isolated from the adult CNS from rodents (24) and later on from humans (25). In the forebrain, neural stem cells were identified based on their ultrastructural characteristics observed by electron microscopy. However, this approach does not allow the purification of live stem cells, and definitive markers for their purification by flow cytometry have yet to be identified (8).

Multipotent neural progenitors are readily and extensively expandable when placed in culture and stimulated with the appropriate growth factors, such as epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) (26, 27). This valuable feature allows adult neural stem cells to be isolated and investigated regarding their functional characteristics and developmental potential. Analysis of intrinsic and extrinsic mechanisms that control the various steps of neurogenesis, including proliferation, survival, fate specification, neuronal migration, maturation and synapse formation, may also be performed (22).

The demonstration of active adult neurogenesis opens possibilities to adult CNS repair after injury or degenerative neurological diseases using cell replacement therapy.

1.1.2.2. Other types of somatic stem cells

Much of the knowledge on stem cells arose from information obtained with hematopoietic stem cells (HSCs). HSCs are present mainly in bone marrow, but also in umbilical cord, peripheral blood and fetal liver, being responsible for the generation of all the mature blood cells in the body (red blood cells, platelets, lymphoid and myeloid cells) (18). In spite of the rarity of these cells (1/10000 to 1/100000 of total blood cells), since 1988 first umbilical cord blood transplantation, umbilical cord and peripheral blood have been used as allogeneic stem cell source (28).

In bone marrow, however, we can also found mesenchymal stem cells (MSCs) (18). These non-hematopoietic stromal cells are capable of differentiating into, and contribute to the regeneration of, mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon and adipose (29). MSCs and MSC-like cells have now been isolated from a wide range of sources other than the bone marrow, including adipose tissue, amniotic fluid, periosteum and fetal tissues, and show phenotypic heterogeneity (30). MSCs are potentially suitable for use in allogeneic and autologous transplantation due to their high proliferative potential, which have drawn researchers' attention into the prospective use of these cells for reparative/regenerative treatment of diseases affecting mesodermal tissues and even in neurological treatments (31).

As continuous sheets of tightly linked cells that constitute the surfaces and linings of the body, epithelia provide a protective envelope against the external environment and regulate water and nutrients absorption and glandular secretions (32). Epithelial stem cells are therefore essential to sustain tissue turnover and repairment of epithelia after injuries. Different populations of epithelial stem cells can generate tissues that display several cellular architectures and functions as distinct as epidermis and intestine.

1.1.3. Induced pluripotent stem cells

In the last decade, engineering of pluripotency into somatic cells by the ectopic expression of transcription factors linked to pluripotency emerged. The resulting induced pluripotent stem cells (iPS cells) were firstly described in 2006 (33) and are the functional equivalents of ES cells in terms of morphology, growth properties, multi-lineage differentiation *in vitro*, teratoma formation and germline transmission. They also express ES cell marker genes (33) and even have the ability to create an entire embryo (from iPS cells injected into tetraploid blastocysts, that alone cannot support somatic development) (2, 34).

Several strategies have been employed to induce this conversion of lineage-restricted cells into a pluripotent state – nuclear transplantation, cellular fusion or explantation, culture-induced reprogramming and infection with virus (3, 35).

Some groups were capable of reprogram mouse embryonic and human somatic cells to produce iPS cells by the introduction of a set of transcription factors linked to pluripotency – the transcription factors Oct3/4, Sox2, Klf4 and Myc, under ES cell culture conditions (33, 34, 36). There are also reports describing the generation of iPS cells from fibroblasts and mesenchymal cells of patients with a variety of genetic diseases with either Mendelian or complex inheritance such as Down syndrome, Huntington and Alzheimer's disease (16, 37).

The development of a method for establishing such immortal cultures not only from healthy individuals but also from disease conditions would offer an unprecedented opportunity to recapitulate both normal and pathologic human tissue formation *in vitro*, thus enabling disease investigation, drug and autologous cell therapies development (16, 38). The iPS can be easily differentiated into neurons (39), although their wide application as models of brain diseases faces significant technical challenges along with the cost and time required to establish and maintain each cell line, which may reduce the ability to compare cells from multiple patients (40).

Since iPS cells represent a patient's own genetic make-up, any tissue derived from the line would necessarily be histocompatible, allowing rejection-proof cell transplantation and eliminating contentious ethical and technical considerations. However, since hES cells were first described in 1998 (15), and human iPS cells only in middle 2006 (33), there can be anticipated some 10–15 years before effective products are developed, thereby launching the era of regenerative medicine (2).

1.2. Mammalian olfactory system

Animals discriminate and recognize numerous chemical signals in their environment with high sensitivity and specificity, which strongly bias their behavior and provide them vital information for survival. The mammalian olfactory system regulates a wide range of multiple and integrative functions such as physiological regulation, emotional responses (e.g. fear, pleasure), reproductive functions (e.g. sexual and maternal behaviors) and social behaviors (e.g. recognition of similar, family and strangers). To achieve such a variety of functions, two anatomically and functionally separate sensory organs, that are sit at the interface of the environment and the central nervous system, are required – the vomeronasal organ (VNO) and the olfactory epithelium (OE) (41).

The vomeronasal organ is specialized in sensing non-volatile chemical compounds (e.g. pheromones), especially concerning the origin of the source. VNO unbranched axons project through an opening in the cribriform plate of the skull to the accessory olfactory bulb (AOB), which in turn transmits sensory information to the vomeronasal amygdala (VA) and then to specific nuclei of the hypothalamus (Figure 5). This accessory organ provides information about the social and sexual status of other individuals within the species (42).

Since humans (and other species) have not retained a fully functional vomeronasal system during adaptation to terrestrial life (41), the olfactory epithelium completes its functions. OE is responsible for the detection of airborne volatile molecules called odorant compounds (or odorants). This neuroepithelium is connected to the next central station of olfactory information processing, the main olfactory bulb (MOB), and then, by mitral cells, to distinct brain nuclei such as the anterior olfactory nucleus (AON) and the olfactory tubercule (OT) (Figure 5).

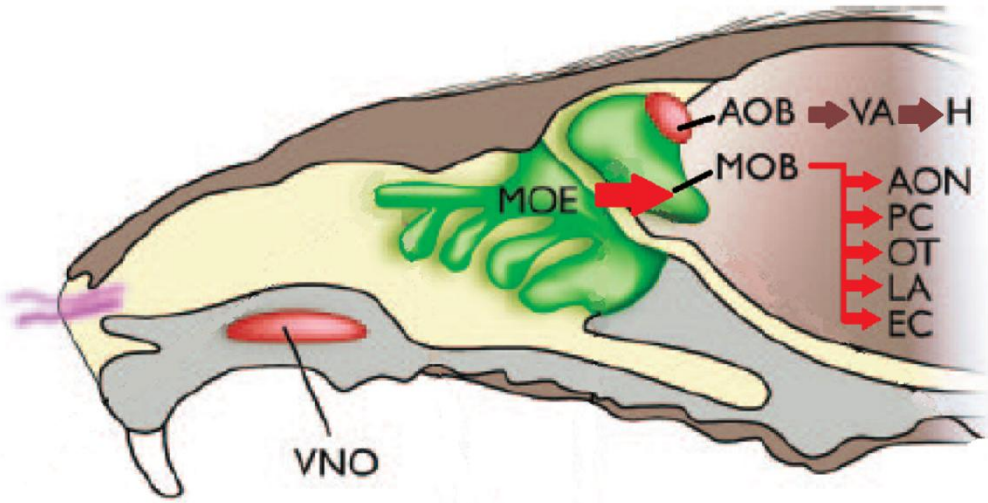

Figure 5 - Schematic view of the two mammalian olfactory systems. In the main olfactory system (red arrows), the olfactory information is transmitted from the main olfactory epithelium (MOE) to the main olfactory bulb (MOB) and then to distinct brain nuclei - anterior olfactory nuclei (AON), piriform cortex (PC), olfactory tubercle (OT), lateral part of the cortical amygdala (LA) and entorhinal cortex (EC). In the vomeronasal system (brown arrows), the information detected in the vomeronasal organ (VNO) is transmitted to the accessory olfactory bulb (AOB), then to the vomeronasal amygdala and finally to the hypothalamus (H). Adapted from (41).

1.2.1. Olfactory mucosa

The human olfactory mucosa (Figure 6) comprises a pseudostratified columnar epithelium resting on a highly cellular lamina propria and occupies 2 cm^2 of the superior portion of the nasal vault, overlying the superior nasal septum, the cribriform plate and the superior part of the superior turbinate (43).

Being the only surface neural cells of the body, but deeply related to the central nervous system, the olfactory mucosa has attracted a renewed interest among researchers as an early marker provider on neurodegenerative conditions, such as schizophrenia, Alzheimer's and Parkinson's disease (44-46). Several neurodegenerative diseases are also partially associated to disorders of smell, not only in the identification and discrimination of odors but also in the odor threshold (47). Besides, olfactory neurosphere-derived cells (ONS) have many advantages over ES and iPS cells, namely the non-requirement of genetic reprogramming and the presence of disease-dependent alterations in gene expression and cell functions (45, 46). Patient-derived olfactory mucosa stem cells shown a shorter cell cycle (48) and faster proliferation (49) in schizophrenia , oxidative stress in Parkinson's disease (50) and altered cell migration in familial dysautonomia (51). There are ongoing studies over the potential of olfactory mucosa as source for autologous stem cell therapy, namely for Parkinson's disease, with encouraging results (52).

Cells from olfactory mucosa can be obtained via septum biopsy through the external naris under endoscopic visualization, local anesthesia and vasoconstriction, a procedure that must be carried out by an Ear Nose and Throat (ENT) surgeon (53, 54). In spite of carrying some inherent theoretical risks, including leak of cerebrospinal fluid, there are no report in literature of severe complications or any adverse effect in the sense of smell after biopsy of the olfactory regions (54).

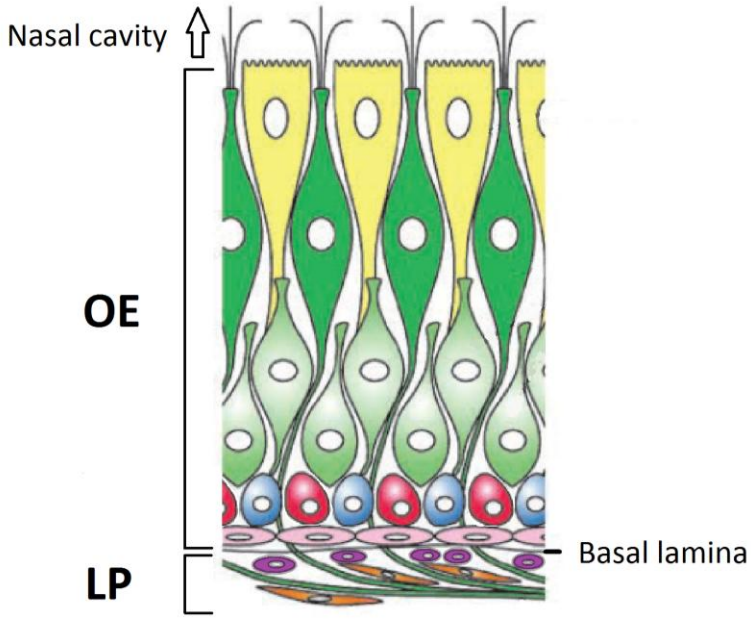

Figure 6 - Schematic representation of histological arrangement of cells in the olfactory mucosa. The olfactory epithelium (OE), adjacent to the nasal cavity, is composed by sustentacular cells (yellow), that surround olfactory receptor neurons (green). The epithelial stem cells, globose (red and blue) and horizontal basal cells (pink), lye on the basal lamina (BL). On the opposite side of the BL there are olfactory ecto-mesenchymal stem cells (purple). The axons of the olfactory neurons that protrude from epithelium into lamina propria (LP) are supported by the olfactory ensheathing cells (orange). Adapted from (11).

1.2.1.1. Olfactory epithelium

Structurally, the olfactory epithelium (OE) resembles the germinative neuroepithelia of the embryo that gives rise to CNS and it is composed by four cell types. From the apical surface to the basal lamina, they are sustentacular cells (SUS), ciliated bipolar olfactory receptors neurons (ORNs), microvillar cells and basal cells (44).

Sustentacular or supporting cells, that are analogous to glial cells of the brain, surround olfactory receptor neurons, probably contributing to the regulation and maintenance of an appropriate ionic environment around the receptor neurons for olfactory transduction to occur

(11). They function as metabolic and physical supports for ORNs (55) and are also responsible for the phagocytosis of dead neurons (56). The ciliated bipolar olfactory receptor cells form the bulk of the OE and project a single dendrite to the surface of the olfactory neuroepithelium and a single axon to the olfactory bulb. The dendrites of these 10 to 20 million bipolar neurons have a thickened end known as olfactory vesicle or knob, that contains non-motile cilia with G-protein-coupled seven-transmembrane domains receptors, where odor molecules bind. The axons cross the basement membrane of the epithelium into the lamina propria, join together into fascicles and nerves, and pass through the cribriform plate to synapse within the olfactory bulb with mitral cells and interneurons, some of which generated by neurogenesis in the SVZ (57).

Microvillar cells are supposed to be a second morphological distinct class of chemoreceptors, however their role in the olfaction has not yet been fully defined (44).

The basement membrane is composed by basal cells that are a population of multipotent stem cells, divided in two distinct types, capable of continuously regenerating olfactory receptor neurons throughout life (44).

1.2.1.2. Olfactory lamina propria

Separated from the epithelium by a basal lamina and lining in the nasal cartilage, the lamina propria (LP) contains axon fascicles, blood vessels, connective tissue and Bowman's glands, whose secretions are probably essential for the olfactory transduction (44, 58).

The axons of the olfactory neurons that protrude from epithelium into lamina propria are supported by an extra line of specialized glial cells: the olfactory ensheathing cells (OECs). These cells guide the regeneration, elongation and migration of non-myelinated olfactory axons, by producing neurotrophic and neurite promoting factors, and ECM proteins. Therefore, several reports described the potential of OECs on the improvement of functional recovery (by remyelination of axons) and axonal re-growth after lesions of the CNS (spinal cord injury) and peripheral nervous system (nerves lesion) (59-61). In addition, a population of multipotent stem cells can also be found in the LP (53, 62).

1.2.2. Neurogenesis in the olfactory system

The cells of the olfactory mucosa are in direct contact with the environment and are greatly vulnerable to damage by toxins, infectious agents or trauma, what makes constant neurogenesis an essential feature for this tissue to maintain critical sensory function.

Therefore, in the adult olfactory system there are at least two germinative zones, where life-long turnover of neurons persists regulated by the same inductive signals (such as neurotrophic factors, retinoic acid and forskolin) (63, 64). One zone is located in the sensory organ, where cell renewal and differentiation are responsible for the replacement of olfactory sensory neurons (65), while the second area resides near the ventricle of the forebrain (41).

The first site of neurogenesis is made possible by the presence of olfactory neuroepithelium stem cells found deep in the olfactory epithelium, near the basal lamina that separates the epithelium from the underlying lamina propria. Globose and horizontal basal cells lie near the basal lamina of the epithelium and have the ability to produce not only neurons but also cells outside the neural lineage (66), such as their ensheatment and supporting cells (67-70). Besides, lamina propria might also provide a source for stem cells in cases of extensive epithelial damage, by the olfactory ecto-mesenchymal stem cells (71).

1.3. Olfactory mucosa-derived stem cells

1.3.1. Epithelium-derived stem cells: HBCs and GBCs

Since the first description of regenerative activity of mice epithelium after axotomy in 1940 (72), there has been a long-running debate over the identity of the true olfactory stem cell population. However, and despite a lack of firmly established stem role, two types of epithelium-derived stem cells are now accepted to contribute to the neurogenic process. Resting in the basal germinal zone and committed to different fates, there can be found globose basal cells (GBCs) and horizontal basal cells (HBCs) (57, 62, 73).

The GBCs represents the major proliferating population, rapidly dividing transit amplifying, that contain round multipotent progenitors with scant cytoplasm (64). They can give rise to olfactory sensory neurons (OSNs), sustentacular cells and, rarely, duct/gland cells and ciliated respiratory epithelial cells (69). On the other hand, HBCs are present as a heterogeneous

population in a single-cell layer below the GBCs, in direct contact with the basal lamina, and are suggested to be more broadly potent, able to generate both neuronal and glial cell types (73). The justification to this greater degree of pluripotency may be the particular ECM environment in which they are included, at the interface between neuronal and glial environments. The majority of adult HBCs are quiescent neural crest-derived cells (55) and, like other non-neural systems, this population divide rarely, probably due to a tight regulation meant to preserve genomic integrity (68).

There is no morphological distinction between HBCs and GBCs. However, direct lineage examination showed that HBCs can replenish GBCs progenitors and indirectly repopulate both neuronal and non-neuronal differentiated lineages of the epithelium after extensive injury (56, 73, 74). HBCs are then presently regarded as 'true tissue stem cells' of the olfactory epithelium, although their cell-lineage and differentiation controlling mechanisms remain unknown (57).

1.3.2. Lamina propria-derived stem cells: OE-MSCs

Since their discovery in 2009 (71), lamina propria-derived olfactory stem cells have been the focus of several studies (53, 62). It is known that exists a strong relationship between these cells and the OE, but their biological function is still unknown. There are, however, some evidences that molecular signaling between LP and OE influences the olfactory pathway formation (75) and that during adulthood, particularly after induced lesion, there can be observed cells migrating from one compartment to another (62, 76).

Lamina propria-derived stem cells were described to be easily expanded and differentiated into neural and non-neural cell types *in vitro* and *in vivo* (66). When compared to bone marrow mesenchymal stem cells (BM-MSCs), these olfactory stem cells display a higher proliferation rate, higher clonogenicity, a susceptibility to differentiate into osseous cells and a low inclination to give rise to chondrocytes and adipocytes, although they can produce fat droplets and calcium deposits (76, 77). Being originated from a neural crest-derived tissue, exhibiting an increased expression of genes involved in neurogenesis and a resident of connective tissue, they are further named olfactory ecto-mesenchymal stem cells (OE-MSC) (62).

Therefore, olfactory mucosa represents a nonepithelial source of multipotent cells that might contribute to the autologous transplant-mediated repair of the CNS injury after nasal mucosa biopsy and transplantation (78, 79). There was the description of hippocampal neuronal networks reestablishment after OE-MSCs injection into cerebrospinal fluid or transplantation of

hippocampal injured mice. OE-MSCs migrated to the affected areas and stimulated endogenous neurogenesis, restored synaptic transmission and enhanced long-term potentiation (80). Besides, other report describe the transplantation of adult human OE-MSCs into the cochlea of a mouse model of early-onset sensorineural hearing loss as a contributor to the reduction of the hearing loss (81). Recently, purified OE-MSCs were described to highly induce oligodendrocytes myelination *in vitro*, with a more robust and efficient effect than BM-MSCs in an eventual transplant scenario (with glial cells) for spinal cord injury (76).

1.3.2.1. Isolation and proliferation of OE-MSCs

In every living individual, olfactory mucosa is easily accessible and it can be safely collected even in humans, by an ENT specialist, without any consequences in olfaction (53, 54).

The procedure of isolating olfactory stem cells that follows olfactory mucosa biopsy is meticulously described (Figure 7) in a previous report (53). Briefly, after biopsy, the explants collected can be used for molecular studies aiming to identify biomarkers in brain diseases. Otherwise, in rodents, the lamina propria could be enzymatically (Dispase II enzyme) separated from the contiguous neuro-epithelium and then stem cells can be obtained by dissociation of the purified LP using enzymatic (Collagenase IA enzyme) and non-enzymatic procedures (mechanical actions). However, if the tissue is human, after the isolation of LP from the underlying OE, it must be sliced into some pieces and inserted under glass coverslips (53). Purified olfactory stem cells can, at this point of the procedure, be used for comparative omics (genomic, transcriptomic, epigenomic, proteomic) studies, aiming to identify molecular markers of CNS diseases (45).

Moreover, these stem cells can then be either grown in large numbers, using a serum-containing appropriate culture medium, as an adherent monolayer, and banked in liquid nitrogen or induced to form three-dimensional spheres on lysine substrate. Presumably, these lamina-propria derived neurospheres, grown in a serum-free medium supplemented with mitogens, represent a population of neural cells in different stages of maturation formed by single, clonally expanding precursors that form tightly packed cellular structures, heterogenous in morphology (spherical, ovoid, and irregular) with a well-defined, phase-bright perimeter (67, 71, 82) (Figure 8). To induce the formation of an higher number of neurospheres, epidermal growth factor (EGF) and basic fibroblast growth factor (FGF2) were used (53), due to their combined mitogenic role in neural stem cells culture (26). The olfactory neurospheres detach from the culture dish and

become free-floating spheres when their diameter reach about 100 µm (45). These neurospheres in suspension must be collected and dissociated enzymatically or mechanically to be re-plated.

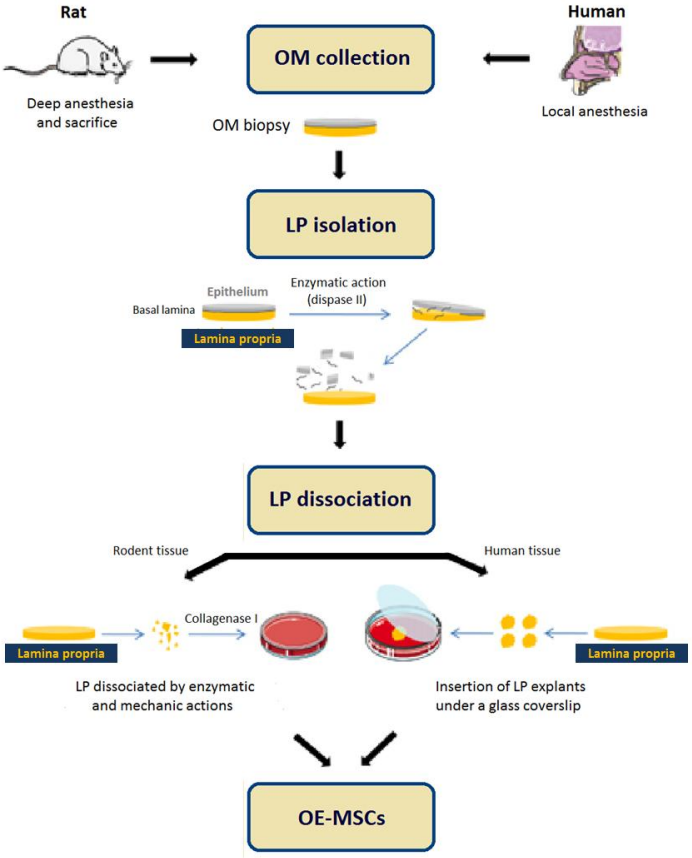

Figure 7 - Overall schematic representation of the olfactory ecto-mesenchymal stem cells (OE-MSCs) isolation. OM – olfactory mucosa; LP – lamina propria. Adapted from (53).

Since the first report on the proliferation and generation of multipotent clones of cells (neurospheres) isolated from adult striatum cells in 1992 (24), those culture conditions - the neurosphere assay - have been used to isolate and characterize other types of candidate stem cell from various other tissues, including skin (83), heart (84) and breast (85).

This neurosphere assay represents a serum-free, selective culture system in which most differentiating or differentiated cells rapidly dye, whereas stem cells and non-stem precursor cells respond to mitogens, divide and form neurospheres that can be dissociated and re-plated to generate secondary spheres or differentiated mature cells, demonstrating self-renewal over an extended period of time (more than five passages) (23, 86, 87). There are descriptions of neurospheres differentiation into neurons and glia, but also cells of non-ectodermal lineage, including developing cardiac and skeletal muscle, kidney, liver and blood (66).

When lamina-propria sphere derived cells are re-plated in a serum-containing medium they give rise to olfactory neurosphere-derived cells (ONS), that grow in an adherent monolayer with a flat, undifferentiated appearance and with a marker phenotype similar to the ectomesenchymal cells derived from primary cultures of OM - GFAP (~50%), β-tubulin III (~10-15%) and O4 (~2-5%) (45, 53, 62). ONS cells represent very practical models for brain diseases because they are not genetically modified and can be quickly generated in large numbers to produce homogeneous populations for functional assays, providing repeatable cell populations for high throughput screening for drug discovery (39).

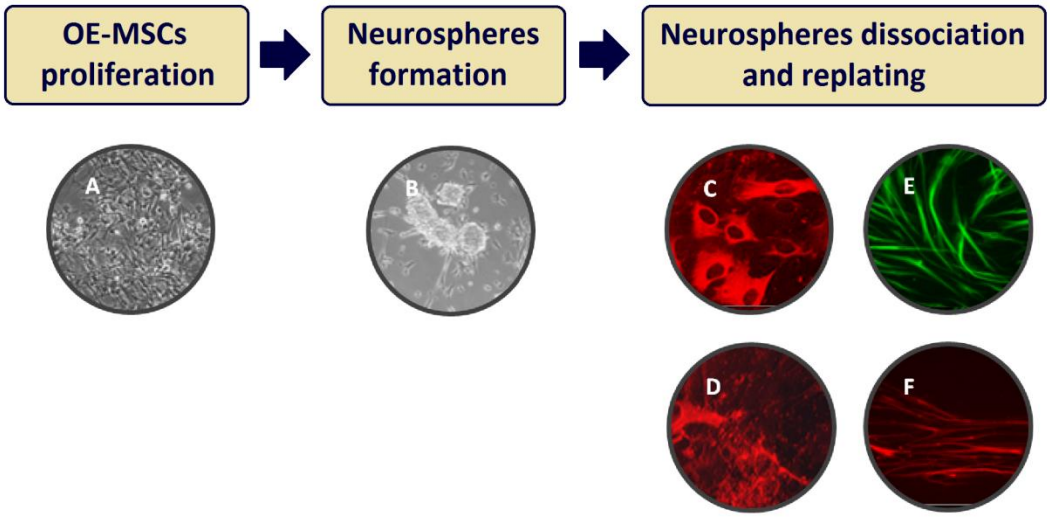

Figure 8 - Culture and differentiation of olfactory ecto-mesenchymal stem cells (OE-MSCs). OE-MSCs proliferate (A) in a serum-containing culture medium. When plated in lysine-coated culture dishes with serum-free medium and growth factors, neurospheres start to appear (B). Free-floating neurospheres can be collected, dissociated and re-plate with the appropriate medium to form olfactory neurosphere-derived (ONS) cells, expressing GFAP (C) and O4 (D), or neuron-like cells, expressing β-tubulin III (E) and MAP2 (F). Real Images Adapted from (53)

On the other hand, there is always the possibility to differentiate the OE-MSCs into neuron-like cells by re-plating them in neurobasal medium supplemented with B27, nerve growth factor (NGF), glutamine and glutamate (53, 66, 88). These β-tubulin III and MAP2–expressing cells are potential candidates for cell therapy uses in brain diseases or trauma (53).

1.3.3. Stem/progenitor markers in the olfactory mucosa

Nestin, an intermediate filament protein expressed in neural stem cells in the brain, was identified as a stemness marker in olfactory mucosa neurospheres as well as primary cultures of olfactory mucosa (62, 66). Stem cells from the lamina propria were also described to express NG2, often referred to as stem cell marker, such as nestin (76).

However, prior to neurospheres formation, that indicates stemness per se, the isolation of olfactory stem tissue is a crucial step. In general, it was not clear in the description of the preparations of olfactory stem cells whether the LP or EPI were present or totally absent. Contamination of OE cultures with cells from the LP and vice versa could be occurring in such studies due to the difficulty in completely removing the LP during tissue dissection, since there were no differences observed in the biology of cells isolated from both LP and OE (71). On top of that, HBCs lack expression of standard neural progenitor markers such as Mash-1 (68, 89), detectable in GBCs (11). A group indicated ICAM-1 (CD54) as marker for HBC stem cells, since they were able to purify these cells by fluorescence-activated cell sorting (FACS) using antibodies to that protein (68). However, ICAM-1 labels other cell types in OM cultures, such as αSMA-positive (smooth muscle actin) smooth muscle cells, what makes it nonspecific (71).

Another study described the purification of HBC and GBC with individual antibodies for each type of cells – BS-1 and GBC-2, respectively, but overlooked LP derived cells (69).

After culturing embryonic rat OM tissue in conditions known to promote neural stem cells proliferation and formation of neurospheres, others identified two distinct populations of spheres and subsequently cytokeratins and Stro-1 as OE and LP specific markers, respectively (71). Such markers were confirmed to be expressed in a report on transplant-mediated repair properties of rat olfactory mucosal sphere-forming cells (82) and in a recent study about the anatomic distribution of the adult human nasal mesenchymal stem cells, Stro-1 was even used to identify LP-derived neurospheres (90). This may represents a way to hereafter improve the standard protocols for isolating nasal olfactory stem cells.

2. AIMS OF THE DISSERTATION

The possibility to investigate the molecular and cellular basis of neuropathologies using stem cells based models systems has been widely explored in the last few years. Recently, both epithelium and lamina propria-derived stem cells isolated from olfactory mucosa have attracted interest among the scientific community. The innovation of these model systems is the advantage of an easily accessible location, high proliferation rate, ability to proliferate in long-term cultures and tendency to differentiate into neuronal-like cells and neurosphere-derived cells (ONS 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:

1. Isolate and proliferate olfactory mucosa-derived stem cells;
2. Induce olfactory neurospheres formation from both epithelium and lamina propria derived stem cells;
3. Differentiate neurospheres into both neuron-like cells and olfactory neurosphere-derived cells (ONS);
4. Characterize both model systems: neuron-like cells and olfactory neurosphere-derived cells;
5. Differentiate neurosphere-derived cells by gradual serum deprivation.

3. MATERIALS AND METHODS

3.1. Isolation and proliferation of olfactory mucosa-derived stem cells

The procedures used throughout the isolation and culture of olfactory mucosa-derived stem cells were based on a recent report by Girard and co-workers (53) and also based on previous work developed at our laboratory (91).

3.1.1. Isolation of olfactory mucosa

Olfactory mucosa was obtained from Wistar female rats (12-22 weeks old) handled according to the European Union directives (86/609/EEC). These rats were acquired from Instituto de Biologia Molecular e Celular (IBMC) and housed under controlled conditions (26˚C under a 12 hour light/dark cycle) with water and food available *ad libitum*.

Animals were sacrificed by rapid cervical stretching followed by decapitation. Skin was removed in order to remove the lower jaw with scissors and a rongeur, and to eliminate the facial muscles on both sides. The bone covering the nasal cavity was removed also with a rongeur, one side at a time, starting from the back incisors. Then, the olfactory turbinates were discarded to expose the olfactory mucosa. Lying on the septum, the olfactory mucosa biopsies were collected and transferred to 35 mm Petri dishes filled with serum-free culture medium (DMEM/HAM F12).

3.1.2. Establishment of primary stem cell cultures

Olfactory mucosa biopsies from both sides of the nasal septum were washed three times in DMEM/HAM F12 to remove the mucus and then incubated in a 35 mm Petri dished filled with 1ml of dispase II solution (2,4 IU/ml), for 1 hour at 37˚C. Then, the lamina propria was carefully separated from the underlying olfactory epithelium, according to their appearance over a dark background. The striped orange/brown lamina propria was transferred to a new 35 mm culture dish with DMEM/HAM F12. The thinner and translucent epithelium was also placed in a 35 mm dish also filled with DMEM/HAM F12.

After isolation, both tissues were cut into small pieces using 25 gauge needles and the culture medium with the floating fragments was transferred to two independent 15 ml tubes. The tubes were then centrifuged 3 minutes at 1000 rpm and the pellets re-suspended in 1 ml of collagenase IA solution (2,5 mg/ml). After dissociate the tissues using a sterile plastic pipette, the tubes were incubated for 10 min at 37˚C. To terminate the dissociation, the tubes were gently

rocked and 9 ml of Ca-free and Mg-free PBS (1x PBS) were added to each one. The tubes were then centrifuged at 1000 rpm for 5 minutes and the supernatants discarded. The cell pellets were re-suspended in 2 ml of DMEM/HAM F12 culture medium (supplemented with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin [Pen/Strep]) and plated in 35 mm cell culture dishes. All cultures were grown under standard conditions at 37˚C and 5% CO_2 and for both tissue types the culture medium was totally renewed every 2 to 3 days. These primary cell cultures will grow until confluence been reached.

3.1.3. Propagation of stem cells

After the establishment of the primary stem cell cultures in 35 mm, they will be divided. The culture medium was aspirated and cells were washed 2 times with 1x PBS. The cells were then incubated with 750 µl of 0,05% trypsin-EDTA solution (Life Technologies) for 3 minutes at 37˚C and after that time 2 ml of complete medium (DMEM/HAM F12 supplemented with 10% FBS and 1% Pen/Strep) were added to the culture dishes. The cells were then re-suspended and plated in a 60 mm plastic culture dish and further incubated at 37˚C and 5% CO_2.

When these plates become confluent they were further divided. The culture medium was removed and the cells were washed with 1x PBS. The cells were then incubated with 1 ml of 0,05% trypsin-EDTA solution for 3 minutes at 37˚C and after that time 3 ml of complete medium were added to the culture dishes. The cells were then re-suspended and plated in a 100 mm culture dish.

Once these stem cells reached confluence, they were further divided as previously mentioned using a 0,05% trypsin-EDTA solution. One single 100 mm plate will be split into 2 100 mm plates. Whenever the cells reached the confluence state, this cells passage procedure was repeated.

3.1.4. Cryopreservation of stem cells

Epithelium and lamina propria-derived stem cells from confluent 100 mm culture dishes were washed twice with 1x PBS and dissociated with 2 ml trypsin-EDTA solution for 3 minutes at 37˚C. After that period of time, 5 ml of complete medium were added to the culture dishes and the cells re-suspended and transferred to a 15 ml tube. After cells centrifugation for 5 minutes at 1000 rpm, the cell pellet was re-suspended in 1 ml of FBS with 10% DMSO and transferred to a

cryopreservation vial. The vial was frozen at -80˚C overnight in a cryo cooler and then transferred into a nitrogen cryotank, correctly identified with the cell type, passage number and freezing date.

3.2. Formation and growing of neurospheres

The primary stem cells can be induced to form neurospheres. Briefly, the culture medium from confluent cultures of lamina propria and epithelium was aspirated and cells were washed with 1x PBS. The cells were then incubated with 2 ml of trypsin-EDTA solution for 3 minutes at 37˚C. After that period of time, 2 ml of complete medium were added to culture dishes. Culture medium with the re-suspended stem cells was then transferred to 15 ml tubes that were centrifuged at 1000 rpm for 4 minutes. After the removal of the supernatants, the cell pellets were re-suspended in 4 ml of supplemented medium (DMEM/HAM F12 supplemented with 1% ITS-X [insulin, transferrin, selenium], 50 ng/ml EGF; 50 ng/ml FGF2 and 1% Pen/Strep) and plated on poly-D-lysine-coated 60 mm cell culture dishes. All cultures were maintained under standard conditions at 37˚C and 5% CO_2. For both cell types (epithelium and lamina propria) a quarter of the culture medium was changed every 2 to 3 days.

3.3. Formation and proliferation of ONS cells

Culture medium with floating neurospheres was transferred to 15 ml tubes and the 2 ml of serum-free medium were added to the dishes to perform fluxes and refluxes with a micropipette, in order to release neurospheres that were still adherents. This suspension with neurospheres was to the 15 ml tubes previously used and the tubes were centrifuged at 1000 rpm for 4 minutes. After the removal of the supernatants, the cell pellets were re-suspended in 1 ml of trypsin-EDTA solution and incubated 1 minute at 37˚C.

After that period of time, 2 ml of complete culture medium were added and then the tubes were centrifuged at 1000 rpm for 3 minutes. After the removal of the supernatants, the cell pellets were re-suspended in 2 ml of serum-containing culture medium and re-plated on poly-D-lysine coated 35 mm cell culture dishes. Olfactory neurosphere-derived (ONS) cells were grown under standard conditions at 37˚C and 5% CO_2. Whenever the cells reached the confluence state, the cells passage procedure described in section 3.1.3 was repeated.

To cryopreserve ONS cells, the protocol described in section 3.1.4 was repeated.

3.4. Culture of ONS cells in defined medium

As mentioned previously, the proliferation of ONS cells is achieved using complete medium. Here we adapt ONS to the absence of serum over 2 weeks through a gradual diminution of the serum percentage (10%, 5%, 2.5%, 1.25%, 0.625% to 0%). The serum percentage was reduced by half every 2 days. Since the cells would not survive in serum-free medium, they were placed in defined media, which consisted of DFN2 (DMEM/HAM F12 with 1% N2) or DFB27 (DMEM/HAM F12 with 2% B27).

In order to analyze the effects of serum reductions and defined media on cell morphology, coverslips with adherent cells were collected at several time points and cells were fixed using 4% paraformaldehyde as further detailed in section 3.6.

3.5. Formation and proliferation of neuron-like cells

Culture medium with floating neurospheres was transferred to 15 ml tubes and then 2 ml of serum-free culture medium were added to the dishes to perform fluxes and refluxes with a micropipette, in order to release neurospheres that were still adherents. This suspension with neurospheres was added to the 15 ml tubes previously used and the tubes were centrifuged at 1000 rpm for 4 minutes. After the removal of the supernatants, the cell pellets were re-suspended in 1 ml of trypsin-EDTA solution and incubated 1 minute at 37˚C. After that time, 2 ml of complete medium were added and then the tubes were centrifuged at 1000 rpm for 4 minutes. After the removal of the supernatants, the cell pellets were re-suspended in 2 ml of Neurobasal medium (Gibco), containing 1x B-27, 0,5 mM glutamine, 0,025 mM glutamate, 1% Pen/Strep, 50 ng/ml NGF and phenol red; and then plated on poly-D-lysine-coated 35 mm culture dishes with an equally coated coverslip. All cultures were grown under standard conditions at 37˚C and 5% CO_2 and a quarter of the culture medium was changed every 2 to 3 days.

These cells were maintained for 37 days and their morphologic evaluation was achieved by taking photos during such time. Morphometric analysis was performed on photomicrographs acquired from live cells using phase contrast illumination in an Olympus IX-81 motorized inverted microscope. Images of 20 fields per culture dish obtained at several time points (1 - 6, 9, 16, 23,

30 and 37 days) using AnalySIS (Olympus) software were analyzed and neurite length was measured using the image analyzer software ImageJ.

3.6. Immunocytochemistry

Cells grown on poly-D-lysine coated coverslips were fixed with a 4% paraformaldehyde solution. Briefly, after the aspiration of the culture medium and 3 washes with serum-free medium, a mixture of 0,5 ml of serum-free medium and 0,5 ml of 4% paraformaldehyde were added to the culture dish for 2 minutes. Then, the medium/paraformaldehyde solution was removed and 1 ml of 4% paraformaldehyde was added to the dish. After 25 minutes and 5 washes (10 minutes each) with 1x PBS, the cells were incubated with 0,2% Triton X-100 in 1x PBS for 10 minutes, for permeabilization. After that period of time, cells were rinsed 5 times with 1x PBS and blocked for 1 hour with a solution of 3% BSA in 1x PBS.

The cells were then incubated for 4 hours at room temperature with the primary antibody diluted in the blocking solution (Table 1). After this time, cells were washed 3 times with 1x PBS. The respective secondary antibody (diluted in the same blocking solution) was added and after 2 hours at room temperature the dishes were rinsed 3 times with 1x PBS and the coverslips mounted in slides using mounting medium with DAPI (VECTASHIELD®). These coverslips were further visualized and imaged using an epifluorescent Olympus IX-81 motorized inverted microscope.

Table 1 – Antibodies used for immunocytochemistry. Dilutions used are indicated for both primary and secondary antibodies.

Target protein	Primary antibody	Secondary antibody
Nestin	Monoclonal Mouse (MAB-353; Millipore) Dilution – 1:100	Alexa Fluor® 488 goat anti-mouse IgG (LifeTechnologies) Dilution - 1:300
β-tubulin III	Monoclonal Mouse (Promega) Dilution – 1:5000	Alexa Fluor® 488 goat anti-mouse IgG (LifeTechnologies) Dilution - 1:300

4. RESULTS

The procedures used throughout the isolation and culture of olfactory mucosa-derived stem cells were based on a recent report by Girard and co-workers (53) and also based on previous work developed at our laboratory (91).

After the isolation of olfactory mucosa-derived stem cells, we established cultures of both epithelium and lamina propria stem cells that were further used in several experiments (Figure 9). Firstly, after these epithelium and lamina propria stem cells were established, they were induced to form neurospheres by culturing them in an adequate medium supplemented with FGF and EGF. The resulting neurospheres were then plated into neurobasal medium containing NGF in order to being differentiated into neuron-like cells or plated in DMEM/F12 complete medium in order to being differentiated into olfactory neurospheres derived cells (ONS). The well-established ONS were then adapted to the absence of serum and cultured in either N2 or B27 supplemented medium to induce some phenotypic changes that are consistent with a neuronal like phenotype. All these procedures are summarized in Figure 9.

Microphotographs were taken for morphologic characterization of all procedures.

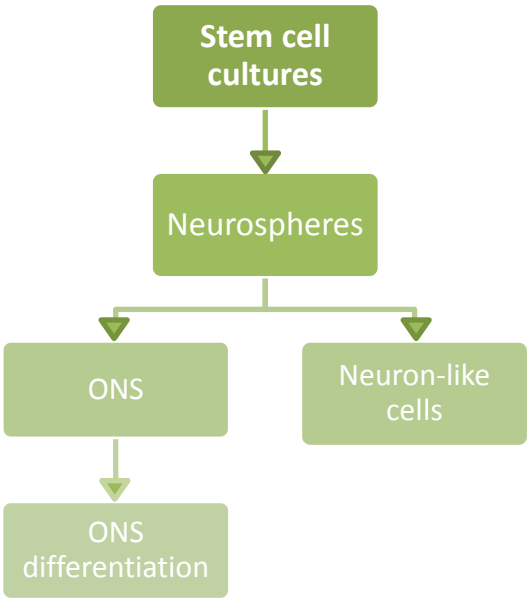

Figure 9 – Outline of the experimental procedures. ONS – olfactory neurospheres derived cells.

4.1. Isolation and proliferation of olfactory mucosa-derived stem cells

Olfactory mucosa was obtained from several Wistar female rats sacrificed by rapid cervical stretching followed by decapitation. After skin, lower jaw and facial muscles removal, the bone covering the nasal cavity was removed in order to discard the olfactory turbinates. This was a crucial step since the underlying olfactory mucosa tends to cling to the olfactory turbinates, what can result in biological material loss. Biopsies of olfactory mucosa were then carefully collected.

After a first digestion with 2,4 IU/ml dispase II solution for 1h at 37°C, epithelium and lamina propria were separated and isolated based on their appearance (color and thickness). The olfactory epithelium was thinner and looked white or translucent, while the lamina propria was darker, striped orange/brown. A further digestion with 2,5 mg/ml collagenase IA solution was performed and after tissue dissociation, epithelium and lamina propria cells were plated separately in 35 mm culture dishes filled with complete medium (DMEM/HAM F12, 10% FBS, 1% Pen/Strep).

As described in literature (53), around day five after isolation, stem cells began to widely invade the culture dishes. However, as soon as day 2, some cells can be seen evading from the tissue fragments, in spite of numerous cells present in suspension and some tissue debris (Figure 10).

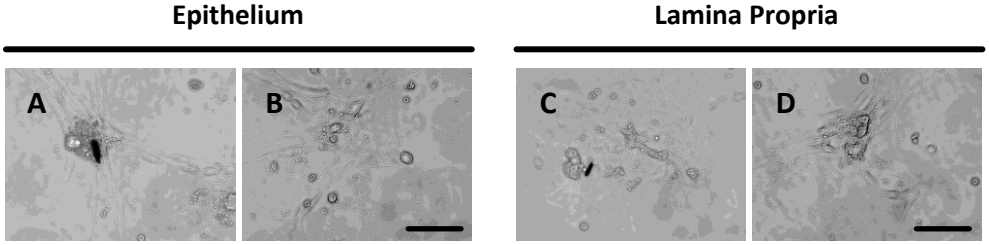

Figure 10 – Olfactory mucosa stem cells (epithelium and lamina propria) after isolation. Biopsies of olfactory mucosa were collected and the epithelium (A and B) was separated from the underlying lamina propria (C and D). Both types of cells were plated in 35 mm culture dishes filled with complete medium, and phase-contrast microphotographs were taken two days after platting. Scale bar = 100 μm

Both types of isolated and proliferated olfactory stem cells – epithelium (EPI) and lamina propria (LP) are considered primary cultures. Whenever these both cell types reached confluence, they were divided and maintained in cultures (according to the passage procedure described in 3.1.3 and passage number (PN) and respective day in culture registered). These cells were kept in culture for long periods (several months), maintaining all the morphological characteristics and the stemness properties. In Figure 11 is represented an example of cells from one of the isolation procedures, kept in culture for 20 passages (PN 20, 4 months).

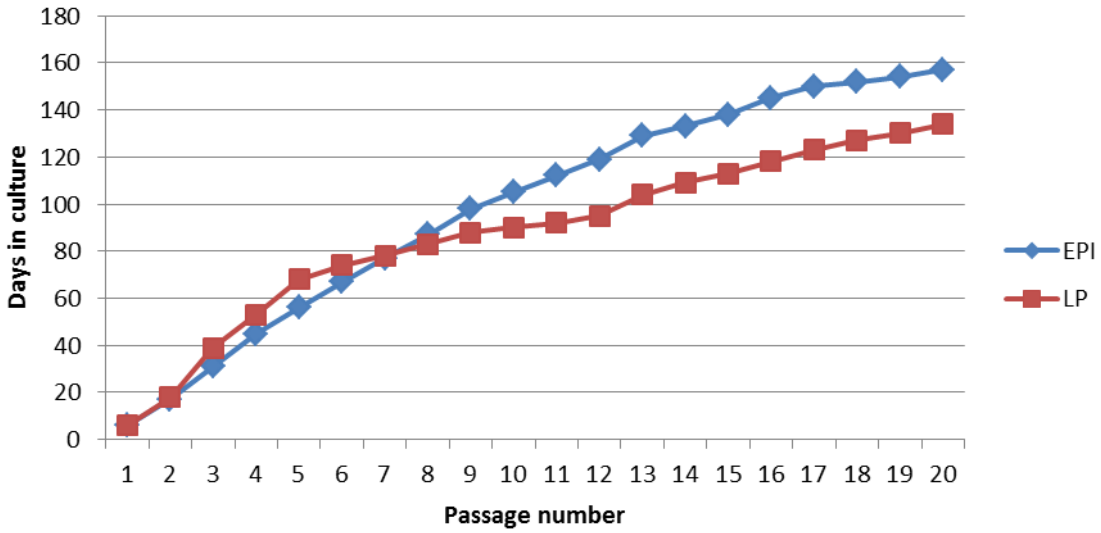

Figure 11 - Passage number and respective days in culture for primary cultures of both EPI and LP. Stem cells from this isolation were kept in cultures for 240 days and almost 50 passages so far. Data shown only for the first 20 passages. EPI – epithelium, LP – lamina propria

Epithelium and lamina propria stem cells were described to have an elongated shape and proliferate as adherent monolayers. To evaluate the efficiency of our isolation and establishment procedures, we examined the morphology of both cultured cells (EPI and LP). Thus, microscopic examination of cultured cells was achieved during the proliferation phase and phase-contrast microphotographs were taken. Images of the primary cultures of both EPI and LP are presented in Figure 12, where we observe that these cultures are mainly composed by elongated adherent cells and there were no significant morphological differences between epithelium and lamina propria derived cells.

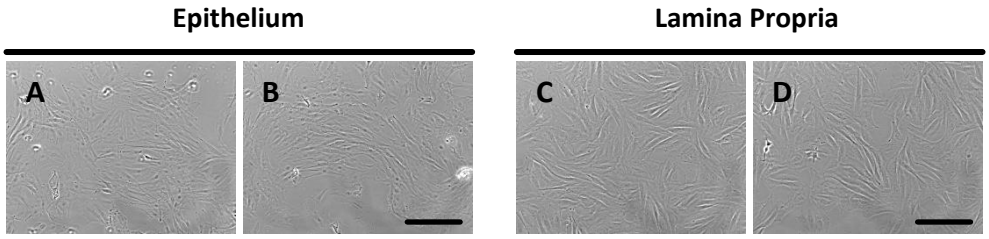

Figure 12 – Olfactory mucosa-derived stem cells morphology. Phase-contrast microphotographs taken from primary cultures isolated from epithelium (A and B) and lamina propria (C and D) cultured in DMEM/HAM F12, 10% FBS, 1% Pen/Strep. Scale bar = 200 µm

To confirm the stemness of the cultured cells, epithelium and lamina propria derived cells were immunolabeled with anti-Nestin monoclonal antibody. This labeling was complemented by nuclei labeling with DAPI. From the immunofluorescence images obtained, we could observe that our cultures expressed nestin, either in epithelium or lamina propria derived cultures (Figure 13). Being nestin a stemness marker, we can assume that we successfully isolated stem cells from both epithelium and lamina propria.

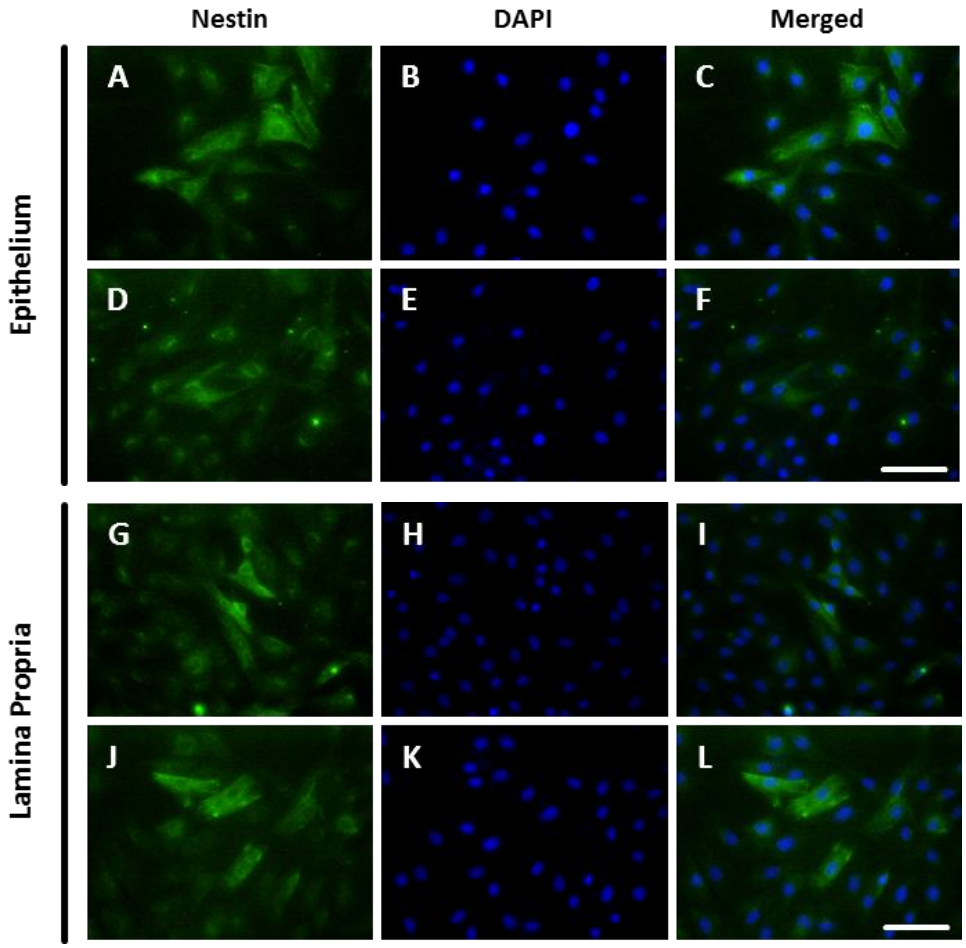

Figure 13 – Nestin immunolocalization in epithelium (A and D) and lamina propria derived stem cells (G and J) isolated from olfactory mucosa. Cell nuclei were simultaneously stained with DAPI (B, E, H and K). The merge images are also presented (C, F, I and L). Scale bar = 100 µm

4.2. Cryopreservation of olfactory stem cells (Epithelium and Lamina Propria)

In order to produce stocks of stem cells from both epithelium and lamina propria to use whenever necessary, both cell types were frozen using FBS with 10% DMSO. After a couple of days, they were unfrozen to evaluate possible effects of the freezing on cell morphology and viability. Three days later, coverslips of the unfrozen cells were collected and fixed to be analyzed by microscopy and the results are presented in Figure 14.

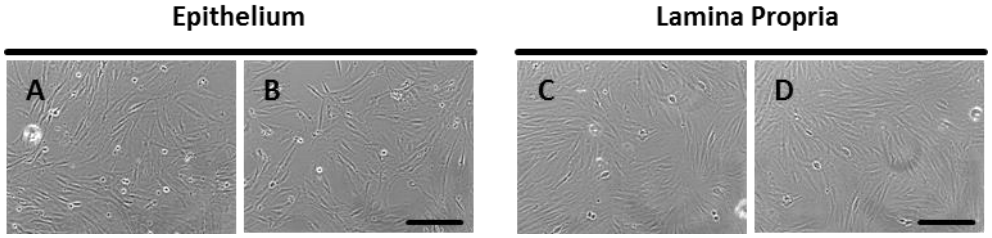

Figure 14 – Morphologic aspect of both cryopreserved epithelium (A and B) and lamina propria (C and D) stem cells. Cells were frozen using FBS with 10% DMSO and unfrozen to evaluate cell morphology. Scale bar= 200 µm

From the photomicrographs taken (Figure 14), it can be assumed that the morphology of the cryopreserved stem cells was not affected by the freeze-thawing cycle. Olfactory stem cells kept their elongated shape, growing as adherent monolayer, exactly as before (Figure 12). Regarding the cellular viability, some dead cells can be observed in both epithelium and lamina propria, although lamina propria culture appears to be more confluent than epithelium.

To confirm the stemness of the unfrozen cells, epithelium and lamina propria derived cells were immunolabeled with anti-Nestin monoclonal antibody. This labeling was complemented by nuclei labeling with DAPI. From the immunofluorescence images obtained, we could observe that our epithelium and lamina propria derived cultures stemness properties resisted the freeze-thawing cycle (Figure 15).

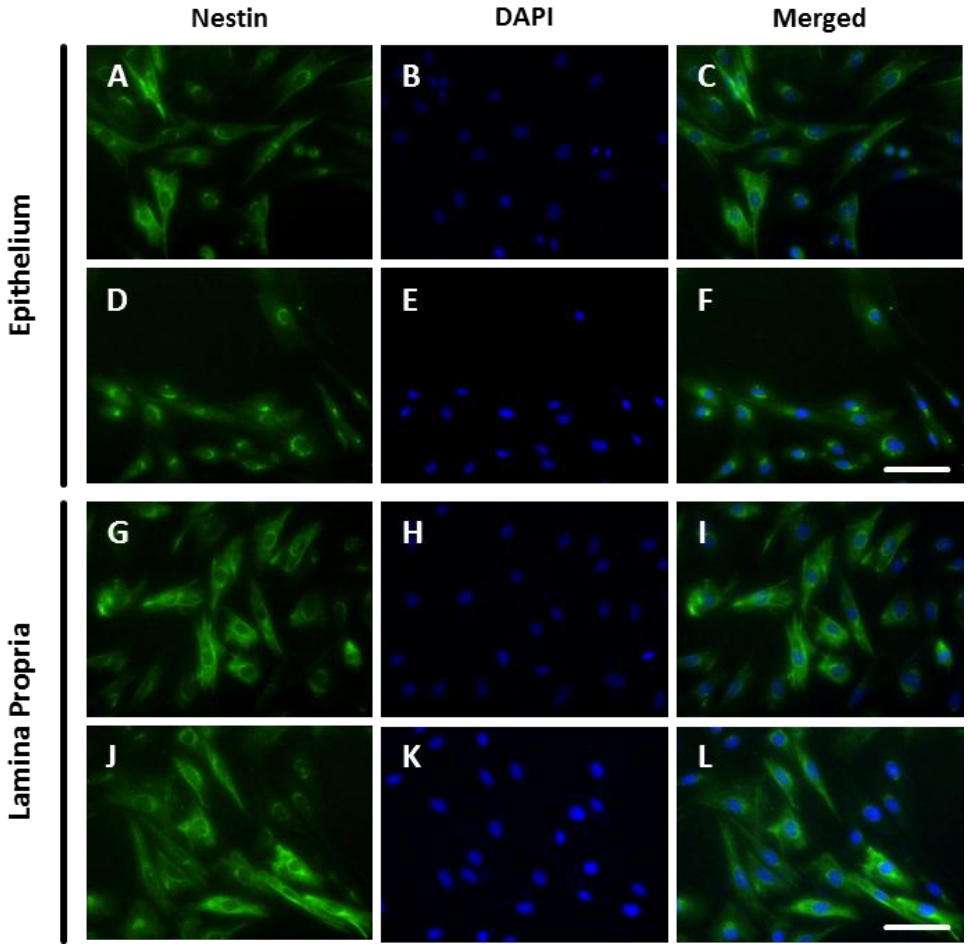

Figure 15 - Nestin immunolocalization in cryopreserved epithelium (A and D) and lamina propria derived stem cells (G and J). Cell nuclei were simultaneously stained with DAPI (B, E, H and K). The merge images are also presented (C, F, I and L). Scale bar = 100 µm

4.3. Induction of olfactory neurospheres formation and growing

After the establishment of primary cultures for both epithelium and lamina propria, we proceed to the induction of neurospheres formation. For that purpose, we platted the olfactory stem cells onto 60 mm culture dishes previously coated with poly-D-lysine. The neurospheres induction was achieved using DMEM/HAM F12 medium supplemented with ITS-X (1%), EGF (50 ng/ml) and FGF (50ng/ml).

To carry further neurosphere formation procedures, we used cells from 70-75% confluent 100 mm culture dishes (45.000-55.000 cells/cm^2) and established several neurospheres culture dishes for both culture type (lamina propria and epithelium). For either culture type a quarter of the medium was changed every 2 days.

As seen in Figure 16 (A, B, K and L), 24 hours after being plated in supplemented medium, olfactory cells from both culture types had already attached to the coated culture dishes and started to organize into clumps. These tendency resulted in cell aggregates that proliferate and give rise to neurospheres around day 3 (Figure 16, E, F, O and P), that continue to develop. Although some neurospheres appeared to float at day 4, olfactory neurospheres were collected at day 5 (Figure 16, I, J, S and T), since a higher number of neurospheres could be collected and used in further experiments. At this time point the majority of cells in the dishes were organized into floating neurospheres, which were spherical and optically dense with a well-defined contour, and the remaining neurospheres attached to the dish surface were easily collected through gentle refluxes of the medium in the culture dish. No differences were observed between lamina propria and epithelium derived cultures and the diameter of the neurospheres were very similar among them (100-150 µm).

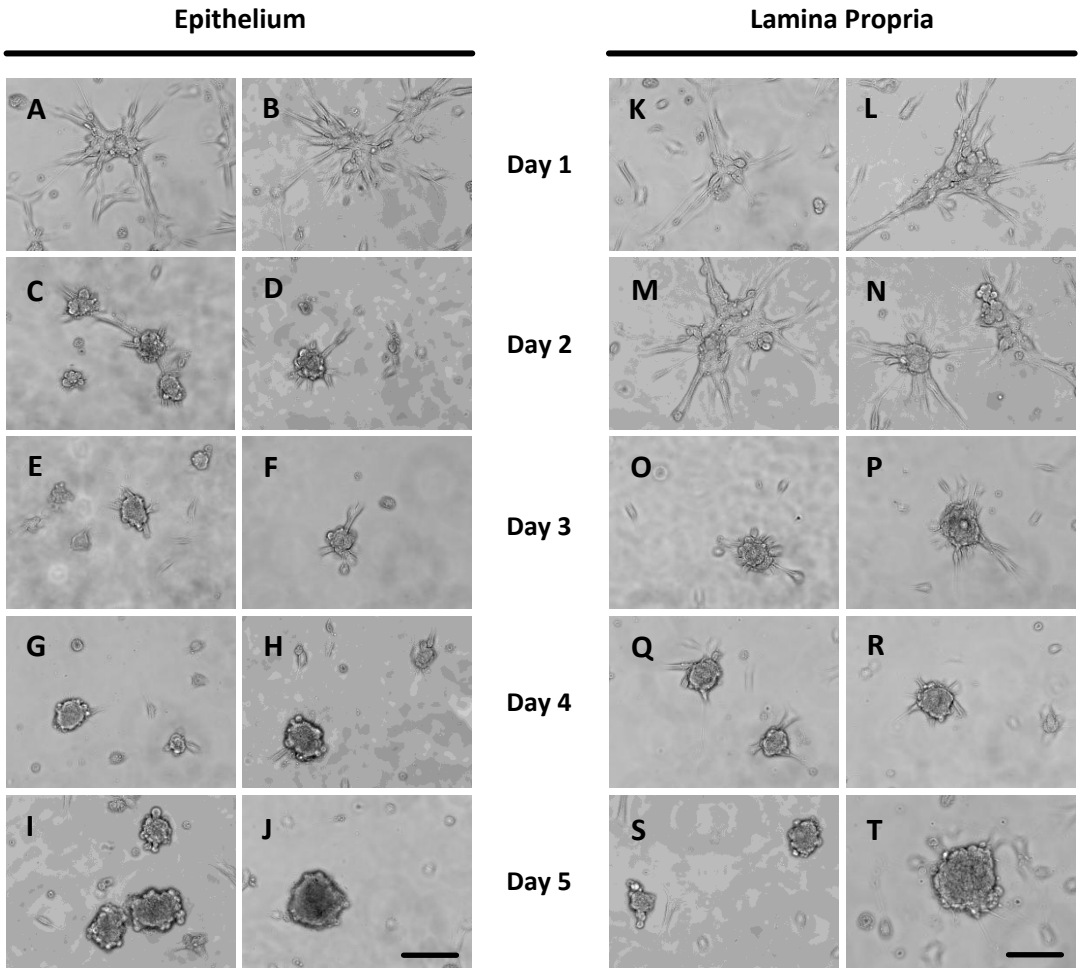

Figure 16 - Induction of olfactory neurospheres formation from epithelium and lamina propria. Cells were plated on 60 mm culture dishes coated with poly-D-lysine. Phase-contrast photomicrographs of epithelium and lamina propria derived neurospheres were taken at day 1 (A, B, K and L), day 2 (C, D, M and N), day 3 (E, F, O and P), day 4 (G, H, Q and R) and day 5 (I, J, S and T). Scale bar = 100 µm

At day 3, coverslips with adherent neurospheres were also collected and cells fixed to perform immunocytochemistry analysis. Neurospheres were then immunolabeled with anti-Nestin antibody (stemness marker) to verify that stem cells are indeed the ones forming neurospheres. In Figure 17, we can clearly observe the tendency of cells to orient themselves into flat clumps (Figure 17, A, B, C, M, N and O), where rapid proliferation occurs (Figure 17, G, H, I, P, Q and R) in order to form free-floating neurospheres.

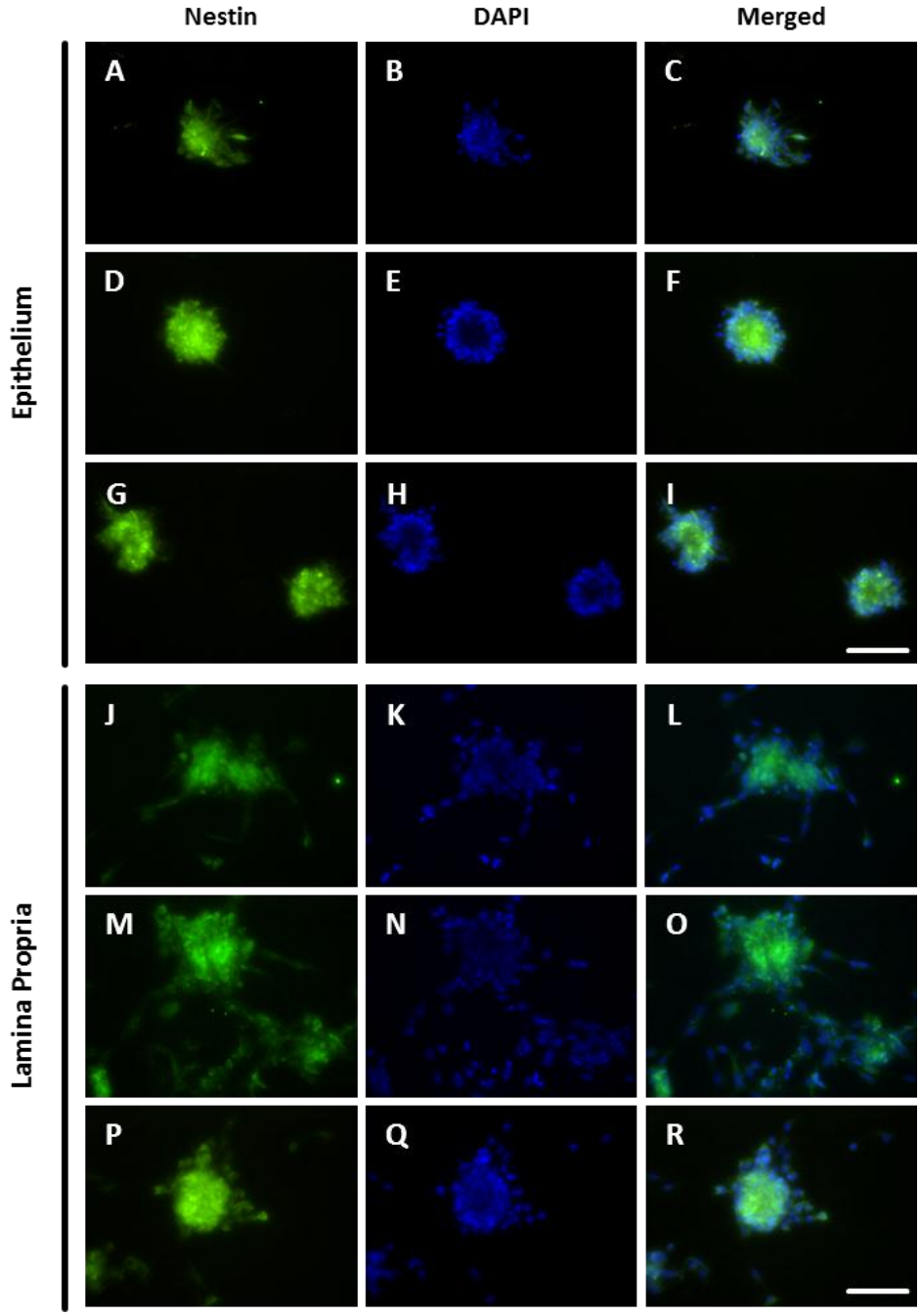

Figure 17 – Nestin immunolocalization in neurospheres from epithelium (A, D and G) and lamina propria (J, M and P). Cell nuclei were simultaneously stained with DAPI (B, E, H, K, N and Q), at day 3. Scale bar = 100 µm

4.4. Neurospheres differentiation into neuron-like cells

The floating neurospheres were collected and further differentiated into neuron-like cells (NLC) using neurobasal medium containing 1x B-27, 0,5 mM glutamine, 0,025 mM glutamate, 1% Pen/Strep and 50 ng/ml NGF. Briefly, after a slight disaggregation trough trypsin action, neurospheres were platted in Neurobasal medium and kept in culture for 37 days. Ten 35 mm culture dishes were established for each culture type (epithelium and lamina-propria). Phase-contrast photomicrographs were taken every day from day 1 to 6 and then at day 9, 12, 16, 23, 30 and 37. For a morphometric analysis of these neuron-like cells, a total of 4.800 images of all conditions were analyzed in terms of cell number and neurite length using ImageJ software.

As seen in Figure 18, 24 hours after being plated, the majority of neurospheres were imperceptible and the cells were well distributed all over the culture dishes. Some processes, similar to neurites, soon arise from the cell bodies (Figure 18). Cells resemble neural cells, being hereafter named neuron-like cells.

After analyzing Figure 18 it is evident that there was an increase in neurite length until Day 6 (Figure 18, Figure 20, Figure 21), although the number of neurites seems to decrease. After Day 6, though such processes are still present, their lengths decrease until Day 37 (Figure 19), when they reach a dimension similar to Day 1.

In terms of cell number (Figure 20, Figure 21), although the mean calculated for the samples appears to decrease, the average deviation for those calculations ends up showing that cell number did not oscillated very much.

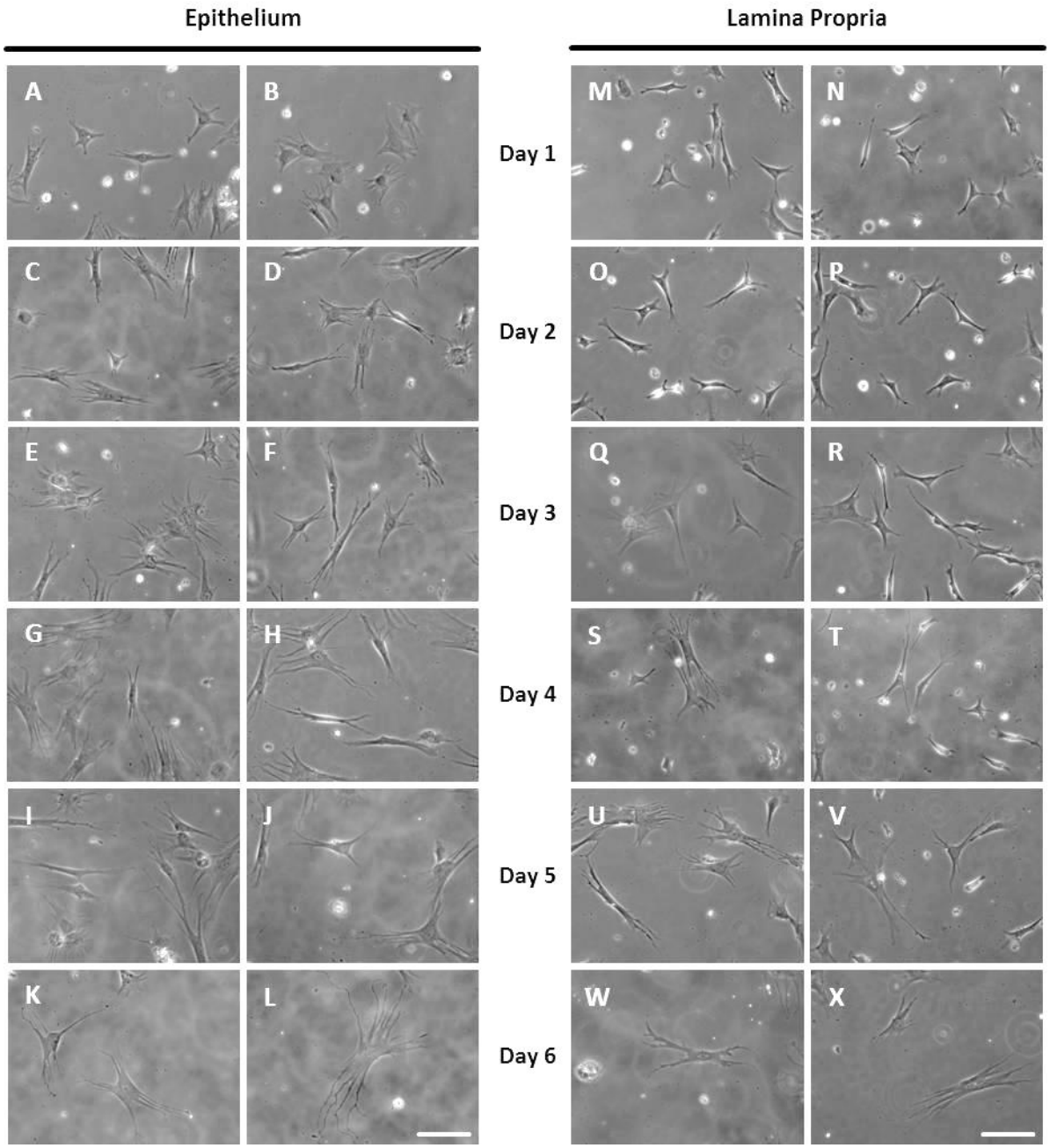

Figure 18 - Neurospheres differentiation into neuron-like cells from day 1 until day 6. Trypsin-disaggregated neurospheres were plated with neurobasal medium on 35 mm culture dishes coated with poly-D-lysine. Phase-contrast photomicrographs of epithelium and lamina propria derived neuron-like cells were taken at day 1 (A, B, M and N), day 2 (C, D, O and P), day 3 (E, F, Q, R), day 4 (G, H, S and T), day 5 (I, J, U and V) and day 6 (K, L, W and X). Scale bar = 100 μm

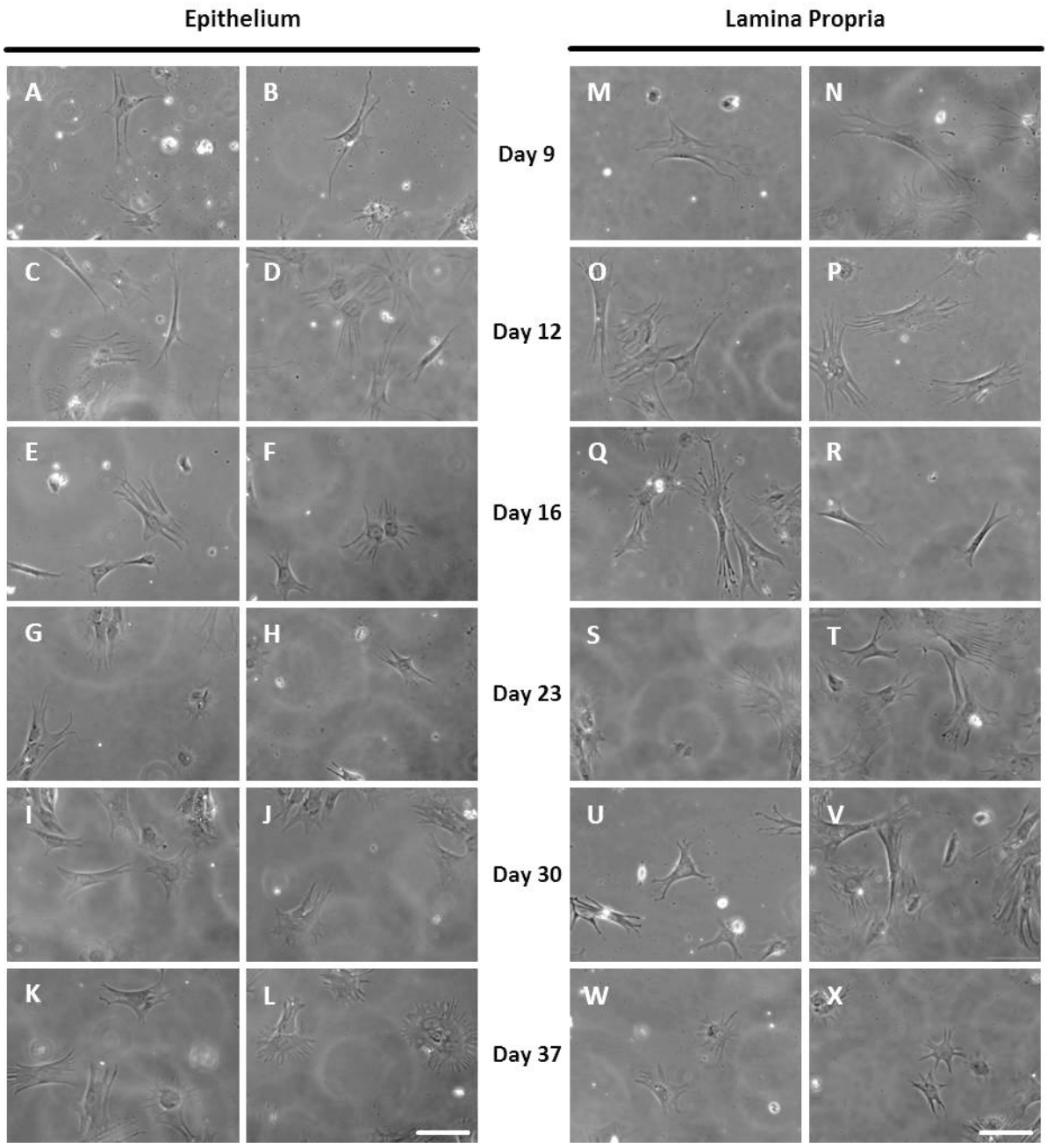

Figure 19 - Neurospheres differentiation into neuron-like cells from day 9 until day 37. Trypsin-disaggregated neurospheres were plated with neurobasal medium on 35 mm culture dishes coated with poly-D-lysine. Phase-contrast photomicrographs of epithelium and lamina propria derived neuron-like cells were taken at day 9 (A, B, M and N), day 12 (C, D, O and P), day 16 (E, F, Q and R), day 23 (G, H, S and T), day 30 (I, J, U and V) and day 37 (K, L, W and X). Scale bar = 100 µm

The morphometric quantitative analysis of all images taken during the time course of differentiation of neurospheres into neuron-like cells is presented in Figure 20 (epithelium) and Figure 21 (lamina propria). In summary, we measured the number and length of neuritis and we counted the total number of the cells. For this analysis, we used the ImageJ software.

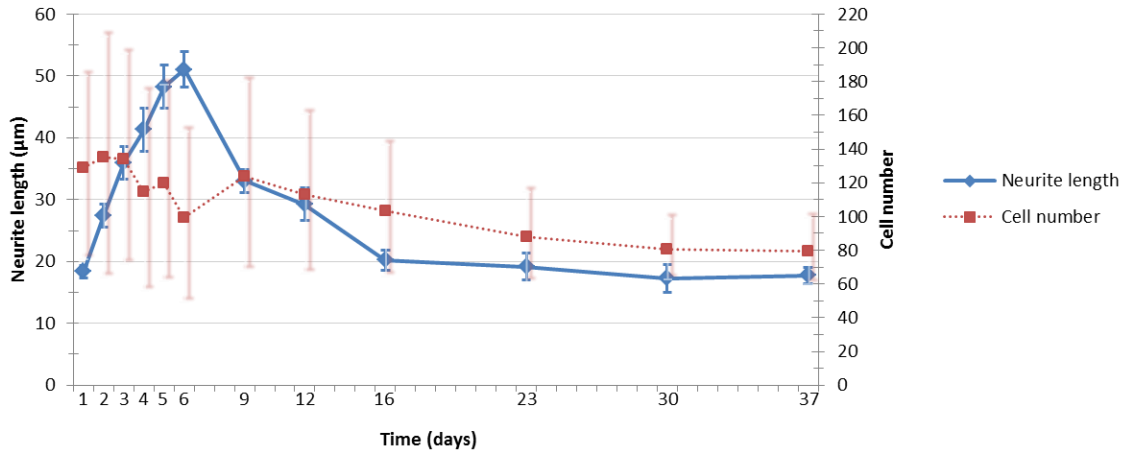

Figure 20 - Epithelium neurospheres derived neuron-like cells morphometric analysis. Images from 10 independent experiments were analyzed in terms of neurite length and cell number. Error bars represent the quotient of standard deviation and square rout of the number of samples (in neurite length series) and average deviation (in cell number series).

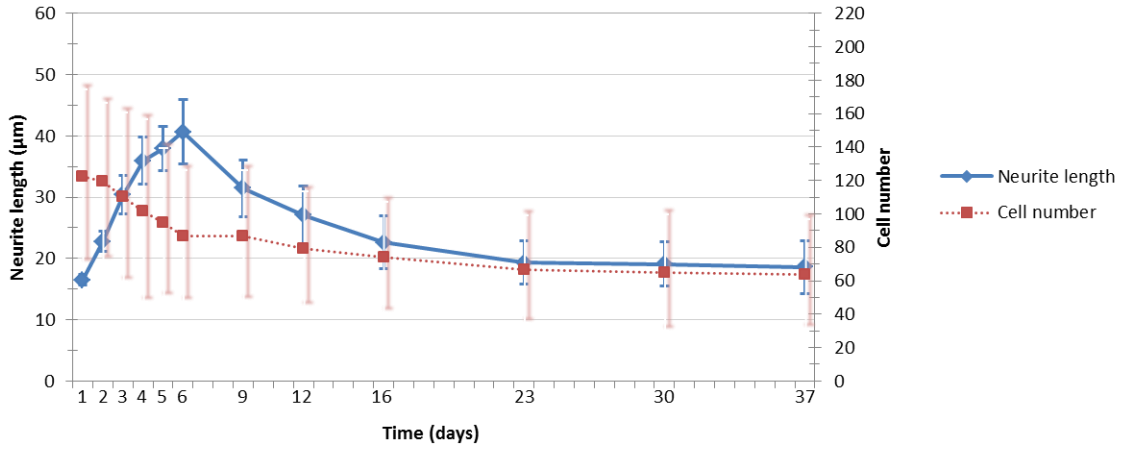

Figure 21 - Lamina Propria neurospheres derived neuron-like cells morphometric analysis. Images from 10 independent experiments were analyzed in terms of neurite length and cell number. Error bars represent the quotient of standard deviation and square rout of the number of samples (in neurite length series) and average deviation (in cell number series).

At day 37, cells were also fixed and further analyzed by immunocytochemistry. Cells were immunolabeled with anti-β-tubulin III antibody (neuronal marker) and nuclei were labeled with DAPI. From the immunofluorescence images obtained (Figure 22) we can observe that our so called neuron-like cells are indeed expressing β-tubulin III, a microtubule element expressed in neurons. No significant differences were observed between epithelium and lamina propria neurosphere-derived neuron-like cells.

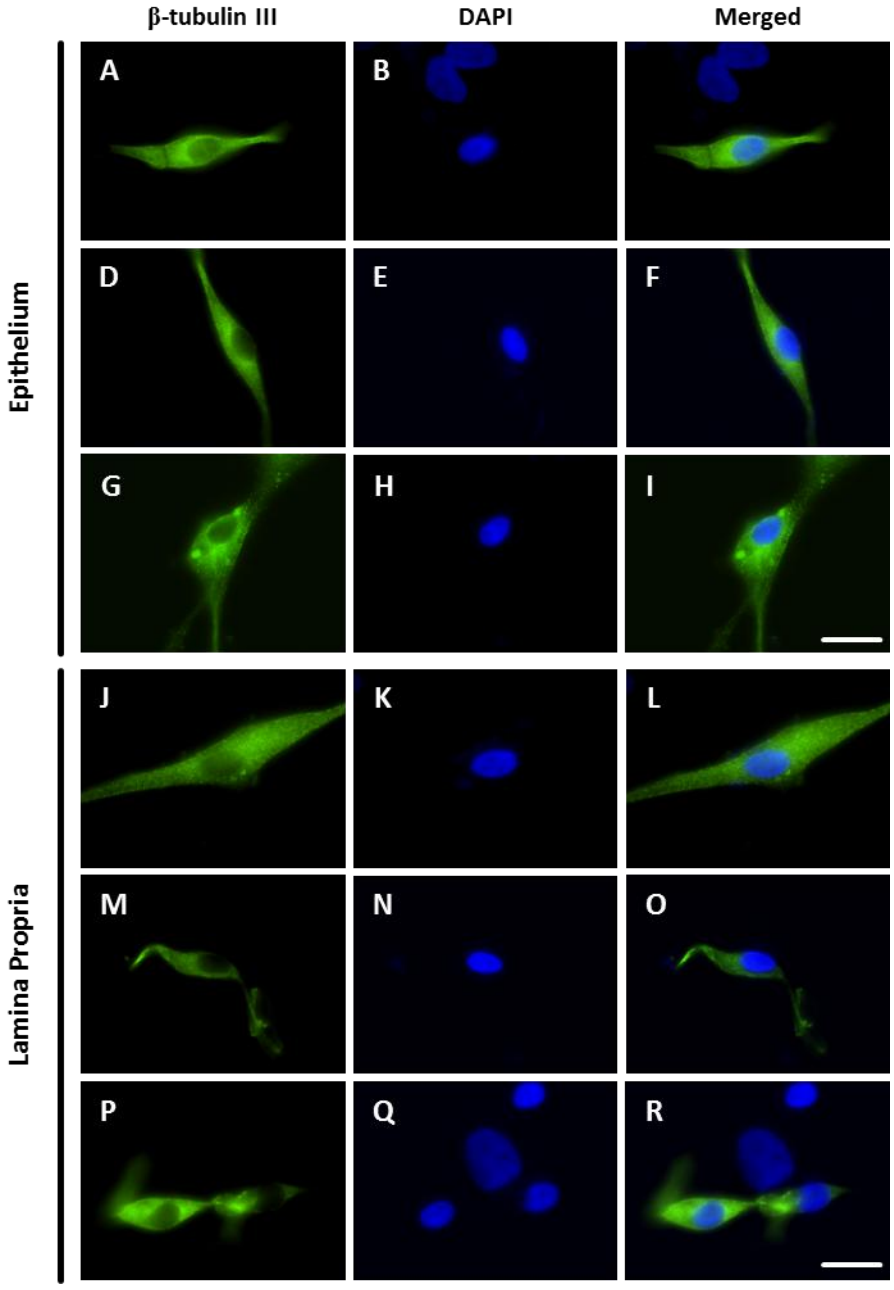

Figure 22 - β-tubulin III immunolocalization in neuron-like cells derived from epithelium (A, D and G) and lamina propria (J, M and P) neurospheres. Cell nuclei were simultaneously stained with DAPI (B, E, H, K, N and Q), at day 37. The merge images are also presented. Scale bar = 20 μm

4.5. Neurospheres differentiation into ONS cells

Additionally, neurospheres of both EPI and LP could also be differentiated into olfactory neurosphere-derived cells (ONS) using DMEM/HAM F12, 10% FBS, 1% Pen/Strep (complete medium). Briefly, after a slight disaggregation trough trypsin action, neurospheres were platted in complete medium on 35 mm culture dishes coated with poly-D-lysine, re-attached to the culture dishes and start to differentiate. Total medium was renewed every 2 days.

A few days after being plated, both lamina propria and epithelium derived neurospheres were flattened and cells were proliferating from the neurospheres periphery. Single cells were also differentiating. At day 7 (Figure 23, A, B, E and F) cells were rapidly proliferating as an adherent monolayer and one week later (Figure 23, C, D, G and H) either epithelium and lamina propria derived ONS were confluent and could be passed into 60 mm culture dishes.

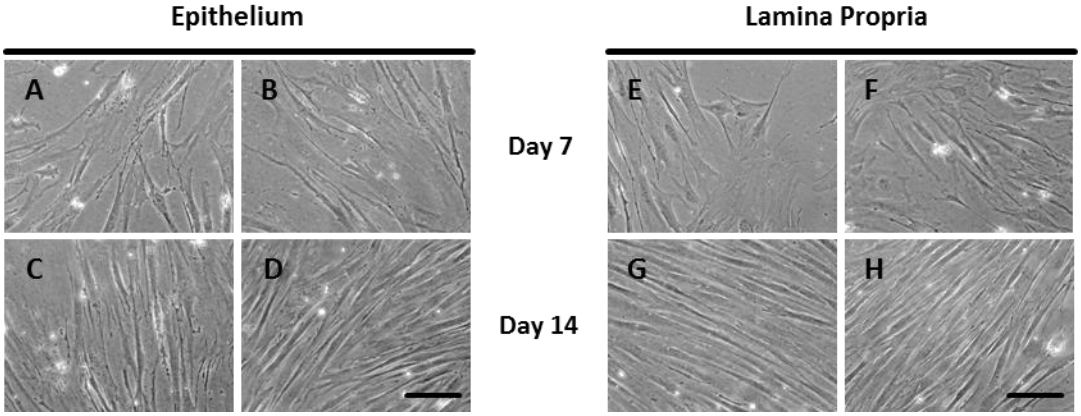

Figure 23 – Olfactory neurosphere-derived cells (ONS). Trypsin disaggregated neurospheres were plated on 35 mm culture dishes filled with complete medium. Phase-contrast photomicrographs taken from ONS of epithelium at day 7 (A and B) and 14 (C and D), and from lamina propria also at day 7 (E and F) and day 14 (G and H). Scale bar = 100 µm

Both culture types were expanded for further experiments and at passage number 10 new morphological analysis was performed, what confirmed the elongated and adherent monolayer growing features of these cells. No morphological differences were detected between epithelium and lamina propria derived ONS cells once again (Figure 24).

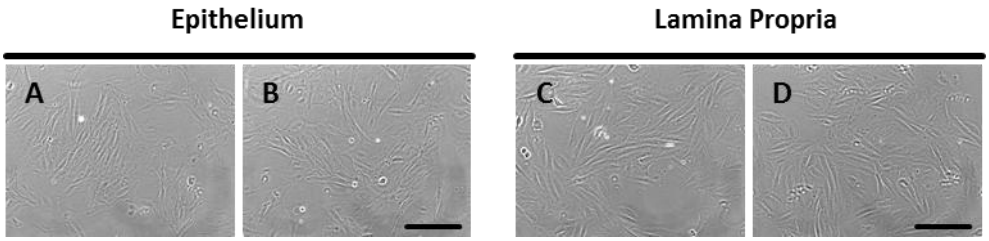

Figure 24 – Olfactory neurosphere-derived cells (ONS) morphology. ONS derived from both epithelium and lamina propria were kept in culture with complete medium and phase-contrast photomicrographs were taken at passage number 10. Scale bar = 200 µm

ONS cells from both culture types were expanded in culture for 14 passages so far, and a similar behavior (growing and days in culture between passages) was observed (Figure 25). Stocks of these cells were also banked down in aliquots after harvest by storage in liquid nitrogen with FBS and 10% DMSO.

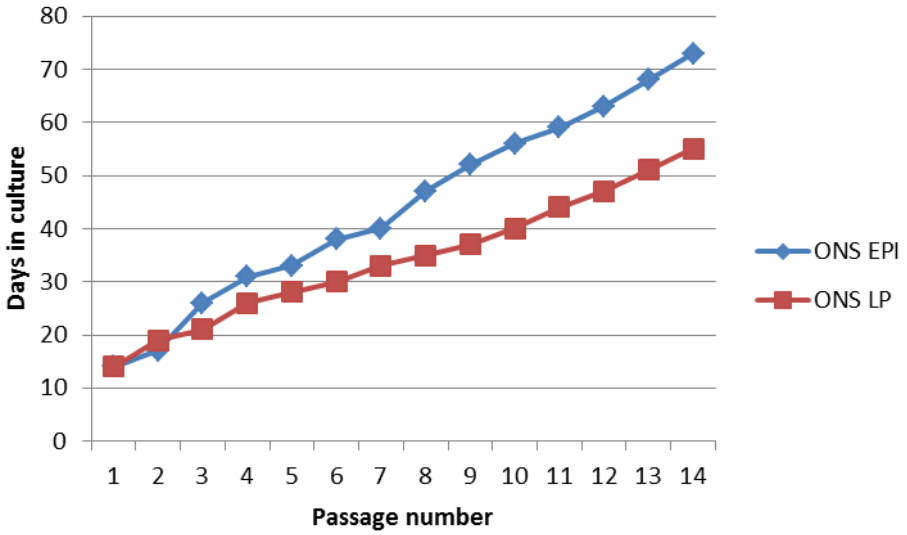

Figure 25 - Passage number and respective days in culture for ONS cells derived from EPI and LP. ONS cells were kept in cultures 14 passages so far. ONS – olfactory neurospheres derived cells, EPI – epithelium, LP – lamina propria

In order to verify if ONS cells express nestin as previously reported (45), epithelium and lamina propria derived ONS cells were immunolabeled with anti-Nestin monoclonal antibody. This labeling was complemented by nuclei labeling with DAPI. From the immunofluorescence images obtained, we could observe that both epithelium (Figure 26, A and D) and lamina propria derived ONS cells (Figure 26, G and J) expressed the stemness marker nestin, although some cells present a low intensity of nestin immunolabeling.

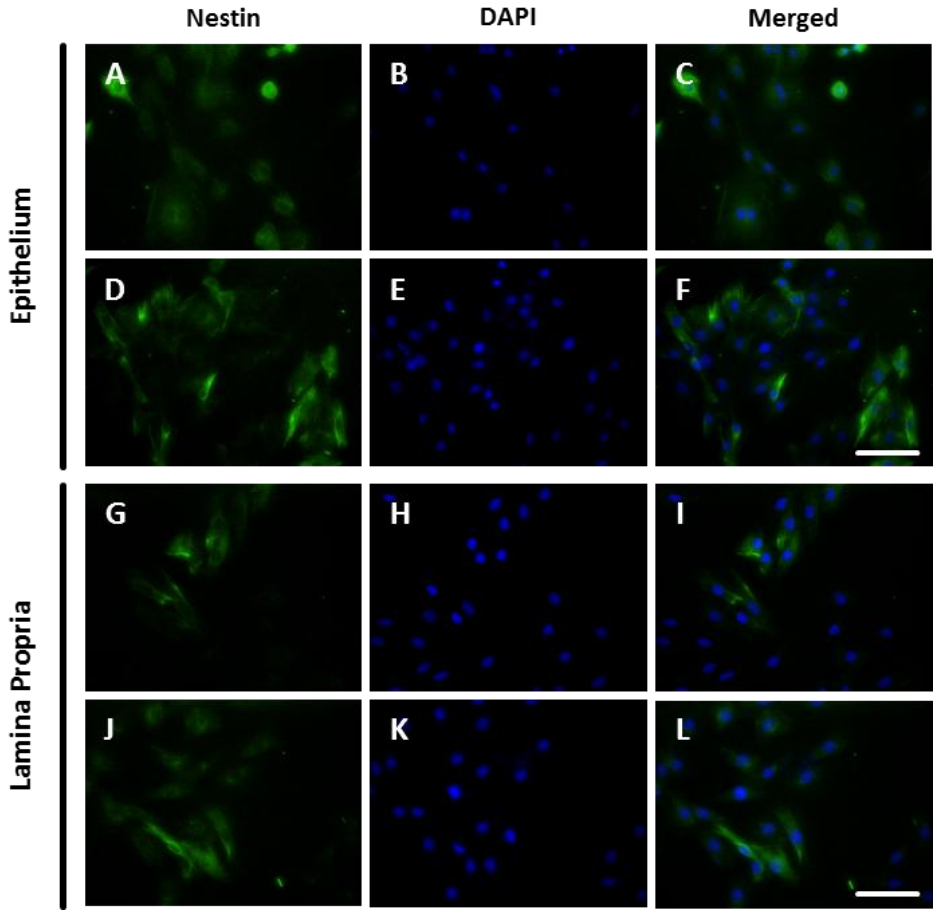

Figure 26 - Nestin immunolocalization in epithelium (A and D) and lamina propria derived ONS cells (G and J). Cell nuclei were simultaneously stained with DAPI (B, E, H and K). The merge images are also presented (C, F, I and L). Scale bar = 100 µm

4.6. ONS cells differentiation

As previously reported (92), ONS cells could be differentiated by gradual serum deprivation and at the end they possess a phenotype very similar to neuronal cells. Therefore, in order to differentiate ONS cells into neuronal-like cells, they were adapted to the absence of serum over 2 weeks through a gradual serum deprivation (10%, 5%, 2,5%, 1,25%, 0,625% to 0%). The serum percentage was reduced by half every 2 days, until they could be placed in defined media, which consisted of DFN2 (DMEM/HAM F12 with 1% N2) or DFB27 (DMEM/HAM F12 with 2% B27).

Serum reduction was started in 70-75% confluent culture dishes from both epithelium and lamina propria derived ONS, since it was predictable that cells would not proliferate as much as usual when the serum was reduced.

In Figure 27, we have the results regarding to gradual serum deprivation. We can observe that ONS cells kept their elongated shape and proliferate even in less serum that usual. After the second serum reduction, when the cells were grown with 2,5% of serum, dishes become confluent and were therefore divided.

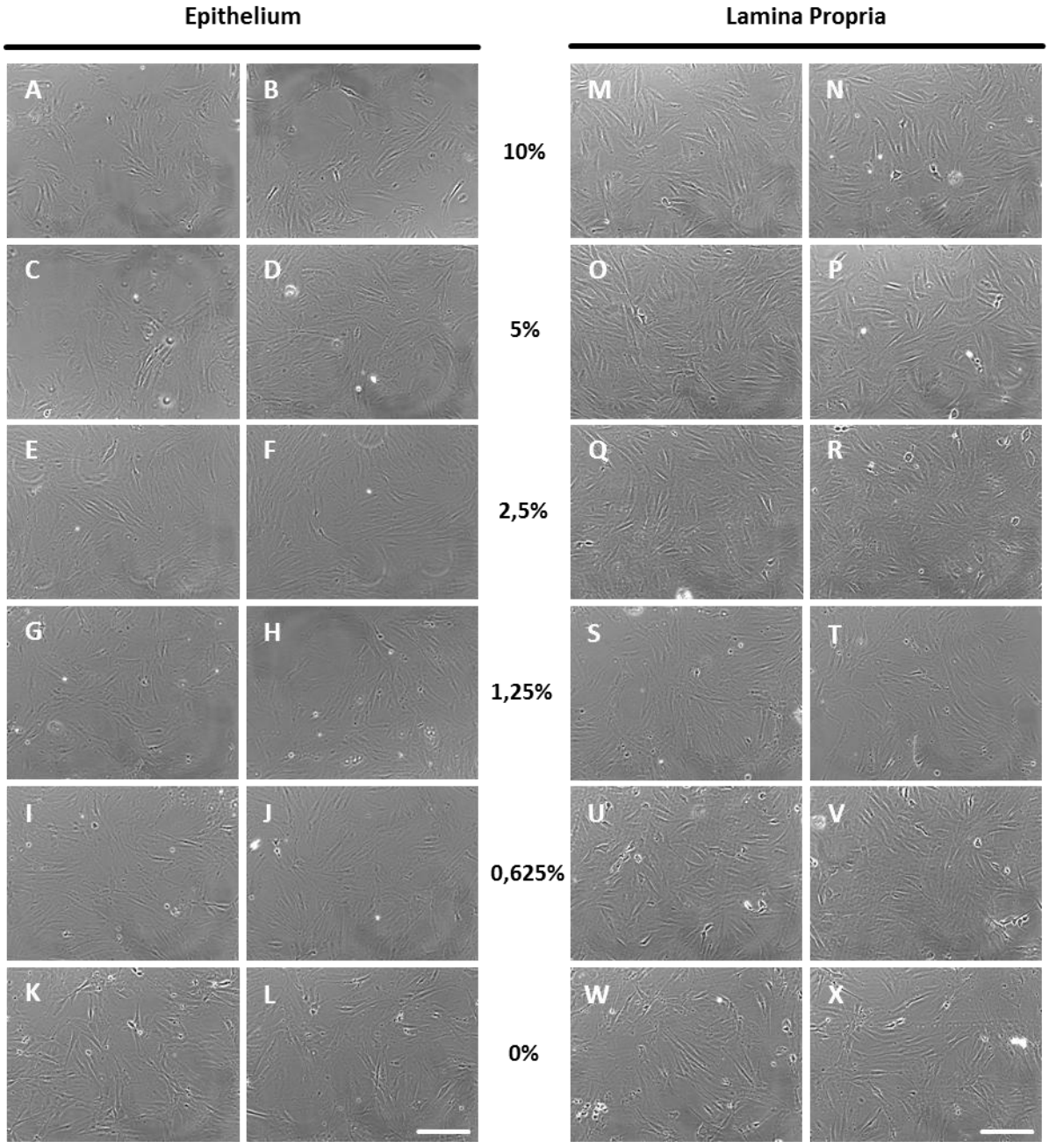

Figure 27 – ONS cells differentiation by serum deprivation. ONS cells derived from both epithelium (A-L) and lamina propria (M-X) were adapted to the absence of serum by gradual reductions in serum percentage (10%, 5%, 2,5%, 1,5%, 0,625%, 0%). Scale bar = 200 µm

For each cell type (epithelium and lamina propria ONS cells) two culture dishes were established. Two days after ONS cells were growing without any serum (0% serum), two new culture mediums were tested. One plate of each cell type was filled with DMEM/HAM F12, 1% N2 and the other with DMEM/HAM F12, 2% B27.

After a few days, cells begin to present a phenotype more similar to neuronal cells using both culture mediums (Figure 28, Figure 29, Figure 30 and Figure 31). They became confluent 4 days after being plated and each of the plates were divided 1:2. However, after that, many cells failed to re-attach the culture dish, especially in the cultures with B27 supplemented-DMEM/HAM F12 medium (Figure 30). Meanwhile, the remaining attached cells not proliferated as expected, and started to die in culture, being discarded during the culture medium renewing procedure. These events were more notorious in lamina propria olfactory neurosphere-derived culture dishes.

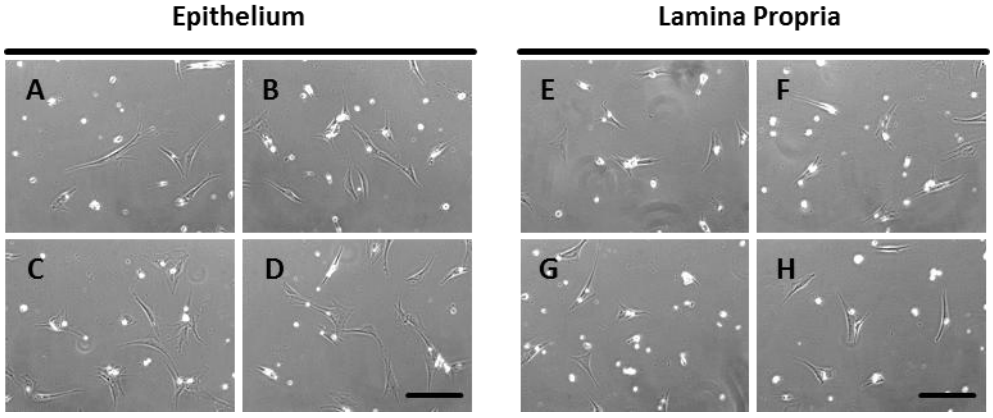

Figure 28 – Olfactory neurosphere-derived cells cultured in DMEM/HAM F12 with N2. Epithelium (A, B, C and D) and lamina propria (E, F, G and H) ONS were cultured in DMEM/HAM F12, 1% N2. Scale bar = 200 µm

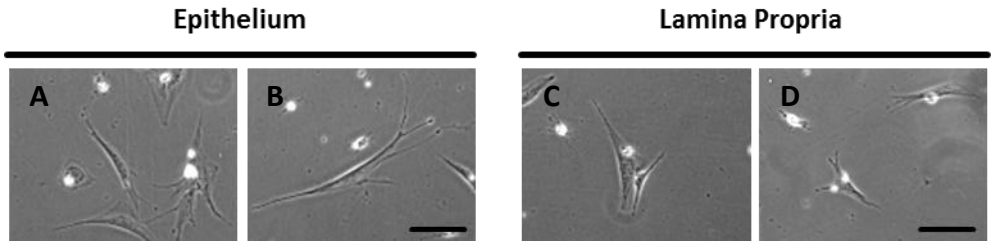

Figure 29 – Detail of olfactory neurosphere-derived cells cultured in DMEM/HAM F12 with N2. Epithelium (A and B) and lamina propria (C and D) ONS were cultured in DMEM/HAM F12, 1% N2. Scale bar = 100 µm

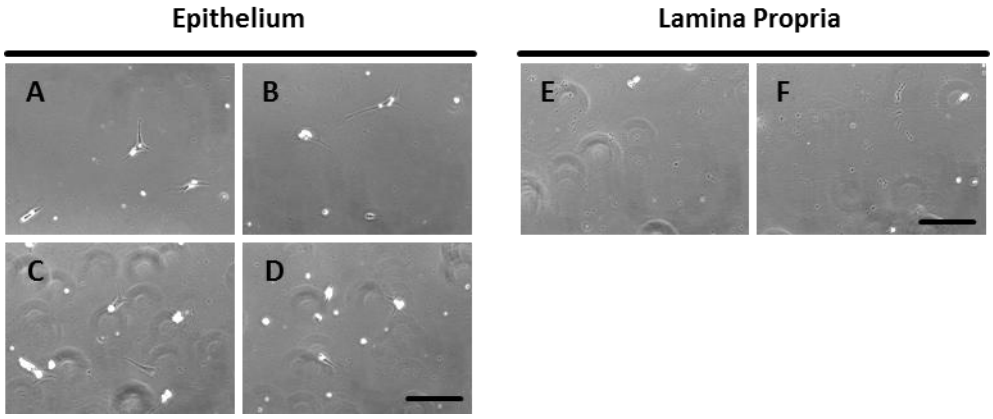

Figure 30 - Olfactory neurosphere-derived cells cultured in DMEM/HAM F12 with B27. Epithelium (A, B, C and D) and lamina propria (E and F) ONS were cultured in DMEM/HAM F12, 2% B27. Scale bar = 200 µm

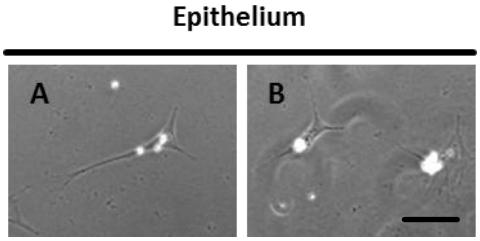

Figure 31 - Detail of olfactory neurosphere-derived cells from epithelium cultured in DMEM/HAM F12 with B27. Epithelium (A and B) ONS were cultured in DMEM/HAM F12, 1% N2. Scale bar = 100 µm

5. DISCUSSION

Neurogenesis continues throughout adult human life due to discrete pools of neural stem cells in the adult brain. Multipotent neural progenitors could present a valuable source of information on neurogenesis process under normal and abnormal conditions, including neuronal migration, maturation and synapse formation, without ethical and legal problems like those associated with ES cells (22). However, their intracranial localization requires a highly invasive surgery for their collection. The olfactory mucosa represents the only source of neuronal cells of the body easily accessible, what explains a renewed interest among researchers. Neurodegenerative diseases such as schizophrenia, Alzheimer and Parkinson, can be studied or even detected through olfactory mucosa cells, that can be easily and non-harmfully collected in adult humans under local anesthesia (54).

Neurogenesis within the olfactory mucosa occurs in stem cell niches in both the olfactory epithelium and the olfactory lamina propria (62). In the epithelium, two populations of stem cells contribute to the neurogenic process throughout life – the horizontal basal cells (HBCs) and the globose basal cells (GBCs). Adult HBCs rarely divide, but can replenish GBCs progenitors, and they can generate neurons and cells outside the neural lineage. Lamina propria-derived stem cells were described to have an high proliferation rate and be easily differentiated into neural and non-neural cell types (71).

After being characterized as olfactory ecto-mesenchymal stem cells (OE-MSC) (62), lamina propria-derived stem cells have been proposed as candidates to autologous transplant-mediated repair of the CNS after injury (78) and model systems to study neuropathologies such as schizophrenia and familial dysautonomia (45, 51).

In the work here presented we intend to isolate and proliferate olfactory mucosa-derived stem cells. In essence, we successfully isolated and proliferated both epithelium and lamina propria-derived stem cells. The accomplishment of this objective was possible due to a recent report by Girard (53) and a previous work performed in our laboratory (91). During this isolation procedure the separation between epithelium and lamina propria is the most difficult step, since the visual barrier between these two tissues is almost imperceptible. Therefore we could have lamina propria biopsies contaminated with epithelium cells and vice versa. However, we strongly believe that our epithelium and lamina-propria cultures are not pure but are highly enriched in each cell type. A recent study suggested an option form the improvement of this isolation step using Stro-1 and cytokeratins as tissue-specific cell markers (71). While cytokeratins are specifically expressed by the epithelium cells, Stro-1 is only expressed in the lamina propria cells. Additionally, cytokeratins are surface protein, which may aid separation. Other report (76)

described the use of a commercial kit to positively select OE-MSCs, that could also be adapted to our procedure workflow.

After isolation, primary cultures of both olfactory mucosa were efficaciously established and proliferated as adherent monolayers of elongated, spindle-shaped cells according to expected. No morphological differences were observed between epithelium and lamina propria derived cultures (Figure 12). Additional nestin immunolabeling of cells from both types of primary cultures (Figure 13) confirmed that we successfully isolated and proliferated olfactory stem cells from the olfactory mucosa biopsies. Thus, we were able to proceed to further experiments using the primary monolayer stem cell cultures isolated.

These primary stem cell cultures soon revealed to be very sensitive cultures and it became clear that the ability to have frozen stocks from both epithelium and lamina propria derived stem cells would be an enormous advantage for further usage of these cells in experimental procedures. Therefore, we tested the effects of a freeze-thawing cycle in the normal morphology and growing features of both stem cells cultures. Both epithelium and lamina propria cultures respond positively to freeze-thawing cycle, since they maintain their elongated, spindle-shaped morphology and still growing as adherent monolayers (Figure 14, Figure 15). Therefore, we conclude that they kept their normal growing properties, morphology and steaminess after a freeze-thawing cycle. This was a great advance for the use of these models system for the study of neuropathologies since we can skip the isolation step that is not always an easy step. Thus, at this moment, we have several aliquots of stem cells frozen, that can be unfrozen whenever necessary to carry with additional experiments.

Since we efficiently proliferated epithelium and lamina propria stem cells we tested their ability to form neurospheres using a specific culture medium. This represents a very important step for the characterization of stem cells since only stem cells have this ability and therefore we efficiently isolate the stem/neurospheres-forming cells from those unwanted non-stem cells. Moreover, in complete medium (DMEM/HAM F12, 10% FBS, 1% Pen/Strep), ensheathing glial cells, fibroblasts, and stromal (sustentacular) eventually established in primary cultures from the isolated stem cells die via apoptosis within 3 weeks of culture (27). Therefore, neurospheres assay are crucial assays for the isolation of stem/neurosphere-forming cells from those unwanted non-stem cells. The crucial step for induction of neurospheres formation is the initial cell density.

Although it has been reported by Girard and co-workers that the ideal initial cell density for neurospheres formation is 16.000 cells/cm^2 (53), other authors suggested an higher cell density: 50.000 cells/cm^2 (58). Previous work of our own laboratory (91) demonstrated that this

higher value is the most favorable plating density to the formation of neurospheres. In our hands, the initial cell density for neurospheres induction was 45.000-55.000 cells/cm^2. Once overpassed this initial cell density problem we efficiently induce the neurospheres formation from both epithelium and lamina propria established primary cultures (Figure 16).

The analysis of the phase contrast microphotographs taken on both cultures during the neurospheres forming assay demonstrate the tendency of stem cells to organize into clumps when plated in FGF2, EGF and ITS-X supplemented culture medium (Figure 16). These results were of paramount importance since we not only confirm once again that we efficiently isolate and proliferate stem cells from both epithelium and lamina propria, but also that we are able to separate those stem cells from the contaminant non-stem cells. Therefore 1-2 days after plating the cells, they start to aggregate and these aggregates generate neurospheres that will be collected to further experiments after 5 days. Characterization of the neurosphere-forming cells was also performed in preparations of day 3-fixed neurospheres. This time point was chosen to avoid the inexistence of fixed cells, once neurospheres start to detach from the culture dishes at day 4. The immunolabeling with anti-Nestin antibody (Figure 17) revealed a high level of nestin in the neurospheres, which confirms that in fact those are indeed the stem cells. As expected we could induce the formation of neurospheres using both epithelium and lamina propria primary cells. The results so far indicate that we efficiently isolate, proliferate and separate the olfactory mucosa stem cells and therefore we are able to differentiate those into neuron-like cells and neurosphere-derived cells, that could be very relevant model systems for the study of neuropathologies.

To assess neurospheres ability to differentiate into neuron-like cells (NLCs) and olfactory neurosphere-derived cells (ONS), neurospheres where collected at day 5, dissociated and re-plated in the appropriate mediums. It is worth mentioning that the dissociation through trypsin action is a crucial step to proceed to differentiation procedures (91), although it was not mentioned in the work of Girard and co-workers (53).

Our original protocol for NLC differentiation (91) that was similar to that proposed by Girard and Co-workers (53) did not include NGF in the differentiation culture medium and failed to differentiate neurospheres into neuron-like cells. According to that protocol, neuron-like cells should appear 2 to 3 weeks after the neurospheres being plated in differentiation medium. However, a previous work reported that few cells resisted for that long in such culture medium and the ones that survive long enough to differentiate died soon after week 3 (91). Thus, based

on other studies (27, 66), we decided to add NGF to the differentiation culture medium to provide an additional stimulus to the differentiating cells.

After being re-plated in neurobasal medium containing 1x B-27, 0,5 mM glutamine, 0,025 mM glutamate, 1% Pen/Strep, 50 ng/ml NGF and phenol red, dissociated neurospheres re-attached to the new dish surface and processes that resemble neurite soon arise from the cell bodies and persisted during 37 days in culture (Figure 18, Figure 19). In fact, our morphometric analysis indicate that there was a significant increase in neurite length until day 6, although after that day there was some retraction in that measure. These results clearly confirm that 6 days after neurospheres differentiation we have neuron-like cells (Figure 18). These results were also confirmed by the immunolabelling of neuron-like cells with anti β-tubulin III antibody that is a neuronal lineage marker (Figure 22). The high expression levels of this protein indicate that we were well succeeded in obtaining neuron-like cells. These results should however be confirmed using additional protein markers of differentiation like synaptophysin and SNAP-25.

When dissociated neurospheres were re-plated in complete culture medium, they easily attached to the new dish surface and olfactory neurosphere-derived cells (ONS) rapidly proliferated as an adherent monolayer (Figure 23). The immunolabelling of those cells with anti nestin antibody have indicated that some ONS cells still express nestin (Figure 26) as their neurospheres ancestors, but in apparently lower levels (less intense staining), as previously reported (45). We established ONS cell lines from both epithelium and lamina propria olfactory neurospheres. Therefore, we established an additional model system valuable for future experiments. Additionally, we have carried out some experiments were we differentiated ONS cells by serum deprivation. During the gradual serum deprivation ONS cells maintain their elongated shape, and after serum removal these cells were cultured in two different culture mediums: DMEM/F12 supplemented with B27 and DMEM/F12 with N2. Our results indicate that the medium with N2 is more adequate for the maintenance of the differentiated ONS cells, with a neuron-like phenotype (Figure 28, Figure 29, Figure 30 and Figure 31). However, additional experiments must me carried in order to optimize this procedure, in order to produce an additional model system based on differentiated ONS cells.

From the results presented we might conclude that we efficiently isolate and proliferate rat olfactory mucosa stem cells from both epithelium and lamina propria that are easily differentiated into neurospheres and therefore separated from non-stem cells. The neurospheres were further differentiated into two valuable models systems that are neuron-like cells and olfactory neurosphere-derived cells. These two model systems were well characterized.

In conclusion, 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.

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7. APPENDIX

I. Olfactory Stem Cells Isolation and Culture

a) Equipment

Centrifuge 5810 R (Eppendorf)

b) Solutions

Serum-free medium [DMEM/HAM F12 (1:1)]

For one liter of medium,

- DMEM (Sigma-Adrich) 6,7g
  Sodium Bicarbonate 1,85g
- F12 (Life Technologies) 5,315 g
  Sodium Bicarbonate 0,588 g

Adjust the pH of each solution to 7.3, mix both solutions and adjust the volume to 1L with deionised H_2O. Filter through a 0.2 µm filter to become sterile.

Dispase II solution (2,4 IU/ml)

For 1 ml of solution, mix 340 µl of dispase (10 mg/ml, Sigma-Aldrich) with 660 µl of serum-free medium.

Collagenase IA reconstitution buffer

- Tricine buffer 0,90 g (50 mM)
- NaCl 2,34 g (400 mM)
- $CaCl_2$ 0,15 g (10 mM)

Adjust the pH to 7.4 and the volume to 100 ml with deionized H_2O. Filter the solution through a 0,2 µm filter and store at 4˚C.

Collagenase IA solution (2,5 mg/ml)

For 1 ml of solution, mix 250 µl of collagenase (10 mg/ml, Sigma-Aldrich) with 750 µl of serum-free medium.

PBS (1x)

For a final volume of 500 ml, dissolve one pack of BupH Modified Dulbecco's Phosphate Buffered Saline Pack (Pierce) in deionized H_2O. Final composition:

- 8 mM Sodium Phosphate

- 2 mM Potassium Phosphate
- 140 mM Sodium Chloride
- 10mM Potassium Chloride

Sterilize by filtering through a 0,2 µm filter and store at 4˚C.

Complete medium

For one liter of DMEM/HAM F12 (1:1), 10% FBS and 1% Pen/Strep,

DMEM	6,7g
Sodium Bicarbonate	1,85g
Pen/Strep (Life Technologies)	5 ml
F12	5,315 g
Sodium Bicarbonate	0,588 g
Pen/Strep	5 ml

Adjust the pH of each solution to 7.3, mix both solutions and adjust the volume to 1L with deionised H_2O. Add 100 ml of FBS (Invitrogen) and filter through a 0.2 µm filter to become sterile.

Poly-D-lysine stock (10 mg/ml)

To a final volume of 10 ml, dissolve in deionized H_2O

Poly-D-lysine (Sigma-Aldrich)	100 mg

Borate Buffer stock

Boric Acid (Sigma-Aldrich)	9,28 g

Adjust the pH to 8.2 and the final volume to 1L with deionized H_2O. Sterilize by filtering through a 0,2 µm filter and store at 4ºC.

Poly-D-lysine solution

Poly-D-lysine (10 mg/ml)	1 ml
Borate buffer	99 ml

Supplemented medium (neurospheres)

For approximately 100 ml of DMEM/HAM F12 (1:1), 1% ITS-X, 50ng/ml EGF, 50ng/ml FGF2 and 1% Pen/Strep,

- Serum-free medium (DMEM/HAM F12) 100 ml
- ITS-X (Invitrogen) 1 ml
- EGF (100 µg/ml, Cell Signaling) 50 µl
- FGF2 (50 µg/ml, Invitrogen) 100 µl
- Pen/Strep 1 ml

Filter through a 0.2 µm filter to ensure sterility.

Neurobasal medium

For 100 ml of neurobasal medium supplemented with 1x B-27, 0,5 mM glutamine, 0,025 mM glutamate, 1% Pen/Strep, Phenol Red and 50 ng/ml NGF,

- B27 (50x, Invitrogen) 2 ml
- Glutamine (200 mM) 250 µl
- Glutamate (10 mM) 250 µl
- Pen/Strep 1 ml
- Phenol Red (Sigma-Aldrich) 200 µl
- NGF (100 µg/ml) 100 µl
- Neurobasal medium (1x, Life Technologies) 96,2 ml

Mix the components of the medium inside a laminar flow chamber to ensure sterility.

DFN2 medium

For approximately 100 ml of DMEM/HAM F12 medium supplemented with 1% N2 and 1% Pen/Strep,

- N2 (100x, Invitrogen) 1 ml
- Pen/Strep 1 ml
- Serum-free medium (DMEM/HAM F12) 100 ml

Mix the components of the medium inside a laminar flow chamber to ensure sterility.

DFB27 medium

For approximately 100 ml of DMEM/HAM F12 medium supplemented with 2% B27 and 1% Pen/Strep,

- B27 (50x, Invitrogen) 4 ml

- Pen/Strep 1 ml
- Serum-free medium (DMEM/HAM F12) 100 ml

Mix the components of the medium inside a laminar flow chamber to ensure sterility.

II. Immunocytochemistry

a) Equipment

Olympus IX-81 motorized inverted microscope (equipped with LCPlanFI 20x/0.40 objective lens)

b) Solutions

4% Paraformaldehyde

- PBS (1x) 100 ml
- Paraformaldehyde 4 g

Filter with a 0,2 µm filter.

0,2% Triton X-100

- PBS (1x) 100 ml
- Triton X-100 200 µl

3% BSA (in PBS)

- PBS (1x) 100 ml
- Triton 3 g