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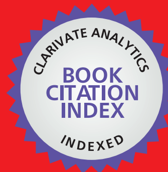

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Pluripotent Stem Cells as an *In Vitro* Model of Neuronal Differentiation

Irina Kerkis[1], Mirian A. F. Hayashi[2], Nelson F. Lizier[1], Antonio C. Cassola[3], Lygia V. Pereira[4] and Alexandre Kerkis[5]

[1]Laboratory of Genetics, Butantan Institute and Department of Morphology and Genetics, Federal University of Sao Paulo,
[2]Departament of Pharmacology, Federal University of Sao Paulo,
[3]Department of Physiology and Biophysics, University of Sao Paulo,
[4]Institute of Biosciences, University of Sao Paulo,
[5]Celltrovet (Genética Aplicada), Ltda.
Brazil

1. Introduction

Embryonic stem (ES) cells are derived directly from inner cell mass (ICM) of mouse or human preimplantation embryos (Evans & Kaufman 1981, Martin 1981, Thomson et al., 1998). They are pluripotent, once they are able to differentiate *in vitro* and *in vivo* into derivatives of the three embryonic germ cell lines: mesoderm, endoderm and ectoderm (Fig. 1). The establishment of protocols for direct *in vitro* differentiation of pluripotent stem cell (PSC) into desirable cell type is extremely important for their use in therapies, for the studies of human diseases, and also for biochemical, toxicological and pharmacological studies (Pederson 1999, Sukoyan et al., 2002, Wobus & Löser 2011). Therefore, focusing on the PSCs use in these studies, many efforts have been devoted to the establishment of stem cells models with a particular emphasis to their *in vitro* differentiation into mature and functional neurons.

The nervous system is the most complex system in the organism and its formation usually involves four stages: specification of the neural cells identity (neural or glia), neural migration and axon formation, synapse formation (with target neurons, muscle or gland cells) and synaptic connection refinement (elimination of axons branches and cells death) (Müller 2006). It is well-known that many genes are involved in the process of neuronal stem cells fate specification (Aiba et al., 2006). This process depends on the specific environment during organogenesis, after birth and during adult life. The temporal and spatial factors are essential for neuronal differentiation, due to the multilayer nature of cortex (Müller 2006). Although numerous publications have reported PSCs differentiation toward neurons, many important questions are not answered yet, especially in respect to the equivalency of the *in vitro* PSCs model and *in vivo* central nervous system (CNS) development. Accomplishments in these directions would represent a crucial starting point for the stem cell therapies and drug discovery. A number of important protocols have been set up for the differentiation of PSCs into neurons, which mainly lead to the coexistence in the culture of differentiated neurons and non-neural cells, together with neural precursors

and undifferentiated PSCs (Okabe et al., 1996, Li et al., 1998, Mujtaba et al., 1999, Baharvand et al., 2007). Most of these protocols are short-lasting, which therefore does not allow a careful analysis of the neurons maturation, aging, and death processes.
In this chapter, we describe principal methods of PSCs differentiation into neurons *in vitro*. Next, we present a method developed by our group, which established a long-term culture of committed neuronal precursors and functional neurons from mouse embryonic stem (mES) cells. In addition, using this long-term protocol we demonstrated the temporal and spatial localization of microtubule-associated proteins, such as, Lis1 (Lissencephaly-1) and Ndel1 (nuclear distribution element-like) in neuronal precursors and differentiated neurons. These both proteins have been shown to be essential for neuronal differentiation during the CNS development. Regardless of the relevance of these proteins for neuronal differentiation, their expression during PSCs differentiation was marginally explored.

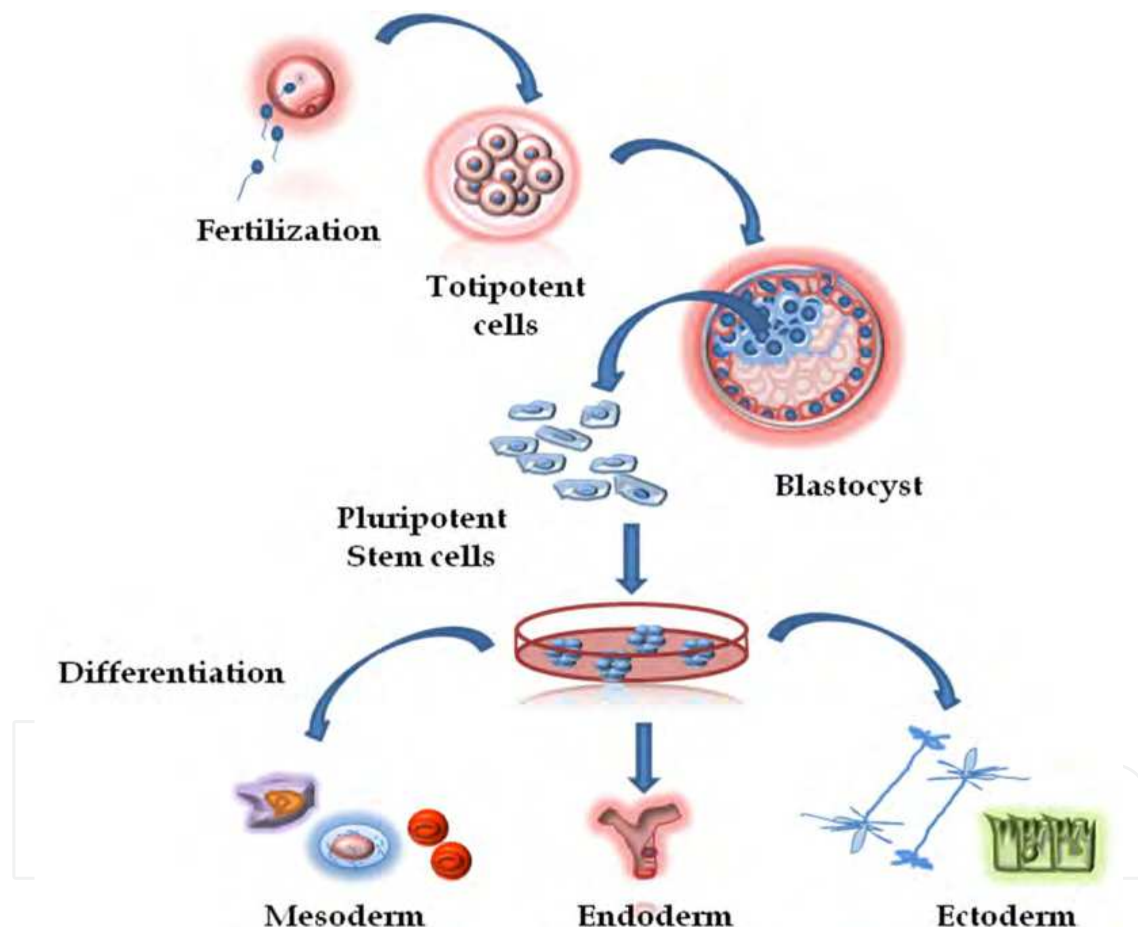

Fig. 1. ES cell isolation and differentiation.

2. Pluripotent stem cells as a model of *in vitro* differentiation

ES cells are powerful biological model, which can provide important information for our knowledge regarding the cell commitment and differentiation during development process (O'Shea 1999, Wobus & Boheler 2005). Multiple methods have been developed in order to induce *in vitro* PSCs differentiation and to obtain the desirable cell phenotype (Baharvand et al., 2004, Keller 2005). It has been found that ES cells are able to differentiate spontaneously

within cell aggregates, when feeder layers and required factors to maintain pluripotency are removed. These aggregates, denominated embryoid bodies (EBs), resemble early post implantation embryos, although chaotically organized inside. It is assumed that EBs formation initiates spontaneous differentiation of ES cells to the three embryonic germ layers (Evans & Kaufman 1981). Innumerous studies have addressed the issue of cell specific differentiation of ES cells. In Figure 2 we summarize the main cell phenotypes, which can be induced to differentiate from ES cells *in vitro* under specific culture conditions. Those accomplishments are the result of a dynamic interaction between knowledge of embryonic development and empirical testing, targeted at reproducing *in vitro* cell specification conditions found in the developing embryo.

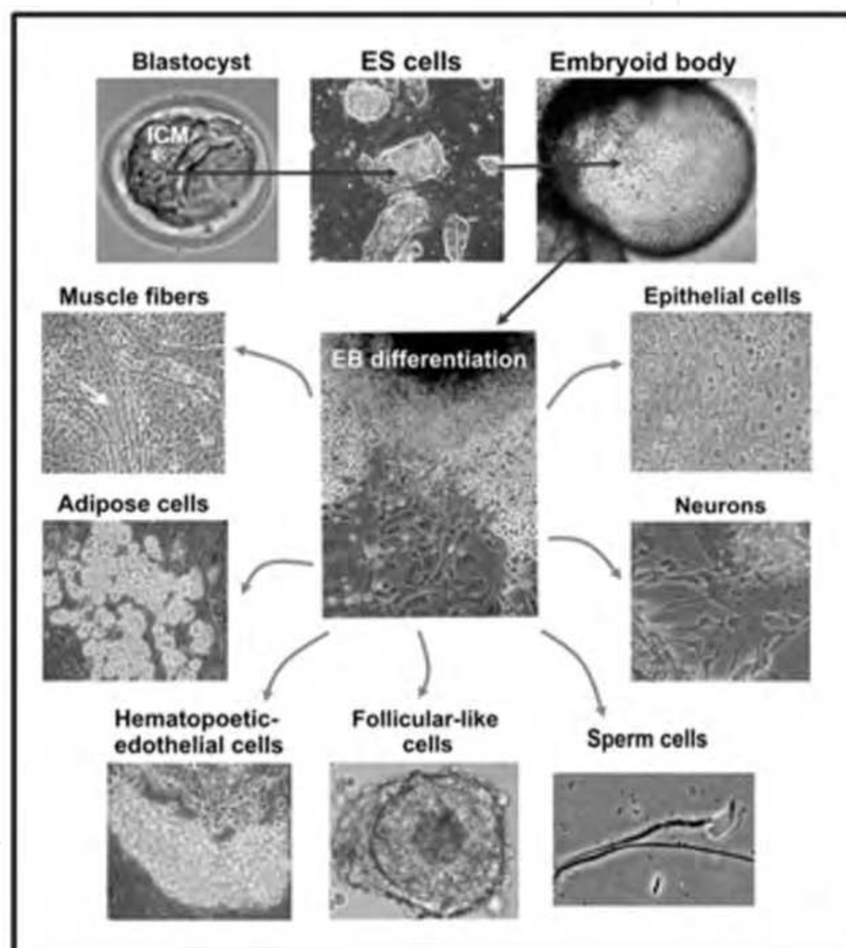

Fig. 2. ES cell differentiation *in vitro*. ES cells are isolated from the ICM of the blastocyst. These cells can be induced to form EBs, which are structures that contain representatives of the three embryonic germ layers. Under the appropriate culture condition, the EBs can be induced to differentiate into several types of cells *in vitro*.

2.1 Mesoderm specification

Mesoderm is the germ layer responsible for the development of muscle, bone, cartilage, blood, and connective tissue. Blood and endothelial cells are the first cell types to form in the developing vertebrate embryo at around six days of gestation. This event leads to the formation of the yolk sac, an extraembryonic membrane composed of adjacent mesodermal

and primitive (visceral) endodermal cell layers, which give rise to blood and endothelial cells (Baron 2001). *In vitro* differentiation of ES cells in EBs allows the generation of blood islands containing erythrocytes and macrophages (Doetschman et al., 1985), whereas differentiation in semisolid medium is efficient for the formation of neutrophilis, macrophages, and erythroid lineages (Wiles & Keller 1991). In an attempt to identify potential inducers of the hematopoietic lineage, researchers indicated Wnt3 (Proto-oncogene protein) as an important signaling molecule that plays a significant role in enhancing hematopoietic commitment during *in vitro* differentiation of ES cells (Lako et al., 2001). The hematopoietic cells derived from ES cells have been characterized by specific gene expression patterns and by cell surface antigens (Wiles & Keller 1991, Wang et al., 1992). However, the most important aspect was to characterize these cells in the functional capacity, by demonstrating long-term multilineage hematopoietic repopulating properties in an animal model (Palacios et al., 1995).

Cardiomyocytes readily differentiate from aggregates composed of mES cells in the presence of high concentration of serum (around 20%), and display properties comparable to those observed *in vivo*: they express similar cardiac gene expression patterns, present sarcomeric structures, and demonstrate contractility triggered by cardiac-specific ion currents, as well as the expression of membrane-bound ion channels. This type of differentiation can develop spontaneously or be induced by differentiation factors including dimethyl sulfoxide (DMSO) and retinoic acid (RA), or small molecules, such as Dynorphin B and cardiogenol derivatives (Fassler et al., 1996). Human ES cells also hold the ability to differentiate into cardiomyocytes, which show similar properties to those derived from mES cells (Kehat et al., 2001). Furthermore, it is well established that ES cells can efficiently differentiate into several other mesodermal cells types, including mesenchymal cell-derived adipogenic (Dani et al., 1997), chondrogenic (Kramer et al., 2000), osteoblast (Buttery et al., 2001), and myogenic cells (Rohwedel et al., 1994). In all of these experiments, the cell type derivation was induced by specific differentiation factors.

2.2 Endoderm specification

Endoderm is responsible for deriving the pancreas and liver. Regarding the therapeutic interest for the treatment of hepatic failure and diabetes mellitus, hepatic and pancreatic cells are of special interest. Thus, since these cells could be derived from ES cells new hope has emerged (Soria 2001). These *in vitro* derived cells showed hepatic-restricted transcripts and proteins, and were able to integrate and to function in a host liver following transplantation (Chinzei et al., 2002). Recently, researchers demonstrated that hepatocyte-like endodermal markers were also detected in ES cell derivatives (Yamada et al., 2002).

The potential use of ES cells for treatment of diabetes was enhanced by the perspective of deriving insulin-producing pancreatic endocrine cells. Researchers at NovoCell, Inc., a biotechnology company in the USA, have developed an *in vitro* differentiation process that mimics pancreatic organogenesis. By directing cells through stages resembling definitive endoderm, gut-tube endoderm, pancreatic endoderm and endocrine precursor, they were able to convert human ES cells to endocrine cells capable of synthesizing the pancreatic hormones insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin (D'Amour et al., 2006). Moreover, in pre-clinical trials, the same group showed that those ES-derived pancreatic cells efficiently generated glucose-responsive endocrine cells after implantation into mice, and those insulin producing cells, in turn protected animals from streptozotocin-induced hyperglycemia (Kroon et al., 2008).

2.3 Ectoderm specification

The embryonic ectoderm is an embryonic germ layer, which can produce various cell lineages during development. Of particular interest, the differentiation of ES cells into neuronal cells was published independently by three groups in 1995 (Bain et al., 1995, Fraichard et al., 1995, Strubing et al., 1995). Gene expression and electrophysiological studies of cell derivatives from PSCs indicated the presence of the all three major cell types of the brain: astrocytes, oligodendrocytes, and neurons (dopaminergic, GABAergic (gamma-aminobutyric-acid-releasing), serotonergic, glutamatergic and cholinergic neurons) (Lee et al., 2000, Rolletschek at al., 2001, Aubert et al., 2002) (Fig. 3). Thus, these studies opened first perspectives regarding ES cell models for the study of neurodegenerative disorders. Human ES cells are also able to generate the neural epithelium (Thomson et al., 1998, Reubinoff et al., 2000, Zhang et al., 2001). However, although neural progenitors derived from ES cells could be enriched and directed to differentiate into mature neurons, astrocytes, and oligodendrocytes (Carpenter et al., 2001), experimental data obtained until recently could not demonstrate the formation of a given neuron subtype (Lee et al., 2000, Rolletschek at al., 2001, Aubert et al., 2002). The possibility of generating neurons *in vitro* signals for a first step towards exploring the therapeutic potential of ES cells for Parkinson's disease (Svendsen, 2008).

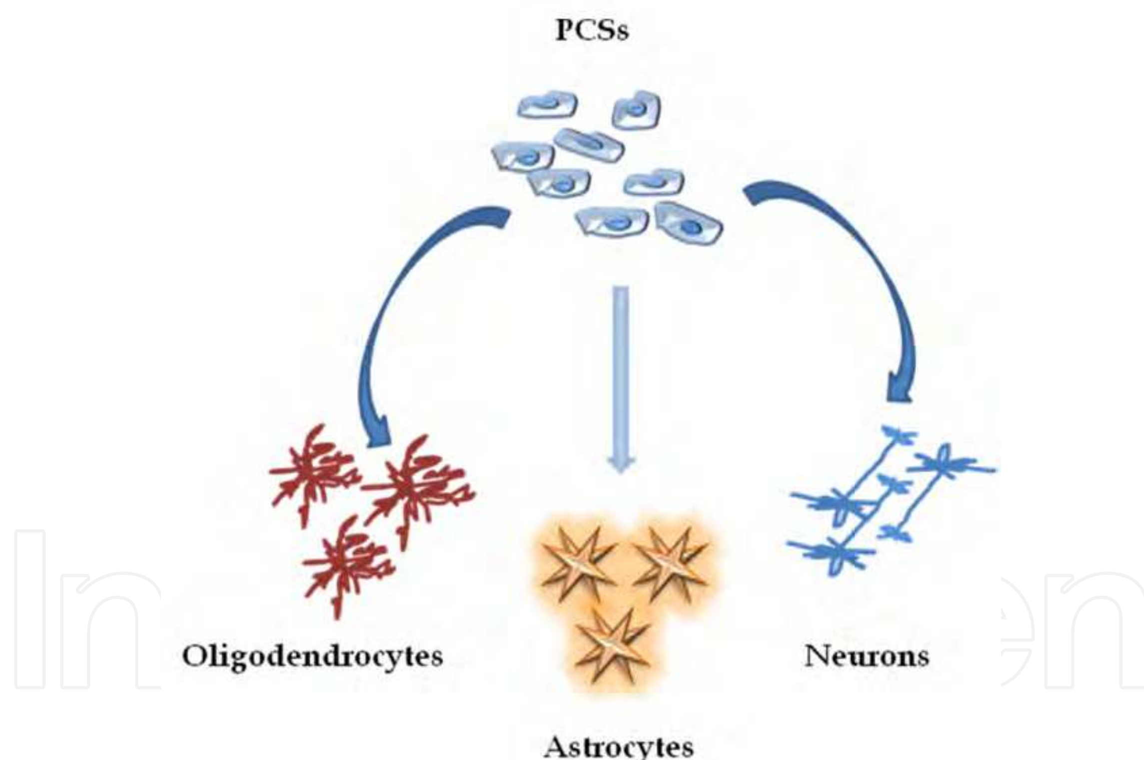

Fig. 3. All three major cell types of CNS derivative from PSCs: oligodendrocytes, astrocytes, and neurons.

2.3.1 Three dimensional (3-D) model of PSCs differentiation

Currently, common protocol comprises three steps: EBs (3-D model) formation, derivation of primitive neuroepithelial cells from EBs and generation of differentiated neural cell types. The most widely used method to induce neuronal differentiation is to enzymatically or mechanically lift the PSCs colonies and place them into low-adherence culture dishes or flasks

without substrate, feeder cells, or mitogens, where they form EBs (Carpenter et al., 2001, Colombo et al., 2006, Baharvand et al., 2007). The culture media formulations for EBs vary significantly between different works (Ng et al., 2005, Yoon et al., 2006). Next, EBs are transferred to serum-free culture media and are plated onto laminin (poly-lysine)-coated dishes in order to generate an adherent culture and to differentiate into neuroepithelial cells. The EBs undergo spontaneous differentiation and the formation of clusters of small elongated cells surrounding a central zone, free of cells, so-called neural rosettes was showed (Pankratz et al., 2007, Pankratz & Zhang 2007). These rosettes resemble the morphology of the primitive neural tube and express early neural marker antigens such as Nestin (type VI intermediate filament (IF) protein) and Musashi-1 (RNA-binding proteins expressed in the CNS), but not markers of more mature neural cells. These rosettes were observed in majority of the studies of induction of neural differentiation of PSCs. PSCs neuroepithelial differentiation method are widely used to generate neural progenitors and mature neural cell types. The neuroepithelial cells obtained by this approach express the neuroepithelial transcription factors, such as PAX6 (Paired box gene 6), Sox1 (Sex determining region Y-box 1), and Sox2 (sex determining region Y-box 2), in about 90% of the total differentiated progenies (Li et al., 2007, Pankratz & Zhang 2007). Neuronal differentiation can also occur without EBs formation. In this case, specific growth factors or the co-culture of PSCs with cells of a particular origin that have been found to produce factors of neuronal cell specification are used to accelerate the differentiation towards one cell type or lineage of interest.

RA is an important regulator of the nervous system development, regeneration and maintenance (Zhang 2006, Maden 2007). Although, rosette formation occurrs during spontaneous *in vitro* differentiation of PSC-derived EBs, addition of RA enhances significantly the yielding of rosettes and mature neurons. Therefore, the predominant number of studies uses RA alone or in combination with other factors, e.g. bFGF (*basic fibroblast growth factor*). Additionally, neural differentiation can also be induced by the withdrawal of bFGF/EGF (*epithelial growth factor*) and exposure to BDNF (*brain-derived neurotrophic factor*), NGF (*neuronal growth factor*) or other factors into the culture medium.

The above described protocol and its modifications commonly produce mixed population of neuronal cells, which contain precursors, neurons and glial cells. This mixed population needs the application of further protocols for selection and enrichment, in order to obtain almost pure population of precursors or neurons, suitable for pharmacological screening or therapeutic applications.

2.3.2 Bi-dimensional (2-D) model of PSCs differentiation

Primary neural stem cells (NSC) can proliferate *in vitro,* forming multicellular floating spherical clusters, commonly referred as neurospheres, which are mainly composed by committed progenitor cells. When adhered on substrate, these neurospheres differentiate into functional neurons (Reynolds & Weiss, 1992; Chojnacki & Weiss, 2008). Our group aimed at developing a protocol for PSCs differentiation into neurons, which resemble the differentiation pattern of NSC-derived adherent neurosphere (AN). This protocol comprises five steps: EBs formation, culturing of floating EBs in the presence of RA, EBs adherence, formation of AN (2-D model), composed by commuted neuronal precursors and generation of neurons from AN. We further referred adherent EBs as ANs. It is worth mentioning that this protocol avoids the formation of rosettes.

The details of 2-D protocol are presented in Figure 4. An enzymatic digestion with trypsin of mES cells were used in order to obtain a feeder-free cell suspension. The mES cells were

plated in culture flask, which allows rapid adherence of feeder cells. The EBs were obtained in low serum (5%) basal culture medium, following routine protocol of hanging drop method. Next, EBs were transferred into low-adherence culture dishes without substrate that allows adherence, and the neuronal differentiation was induced by the addition of RA (at final concentration of 0.1 μM). The EBs were maintained under non-adherent serum-free culture conditions (neurobasal (NB) medium supplemented with B27), for additional 4 days. Next, RA was removed and the EBs was transferred to poly-lysine treated plastic dishes in order to form ANs. The ANs were maintained in serum-free conditions for additional 7 days. At this moment of neuronal differentiation, outgrowth of neuron-like cells on the periphery of ANs was clearly observed (Fig. 5). These ANs were caught in small pieces and mechanically transferred into another Petri dish. After 3-4 days, these small ANs start to produce outgrowth of neurons. This process of ANs mechanical splitting and transfer can be repeated several times continuously producing ANs and neurons.

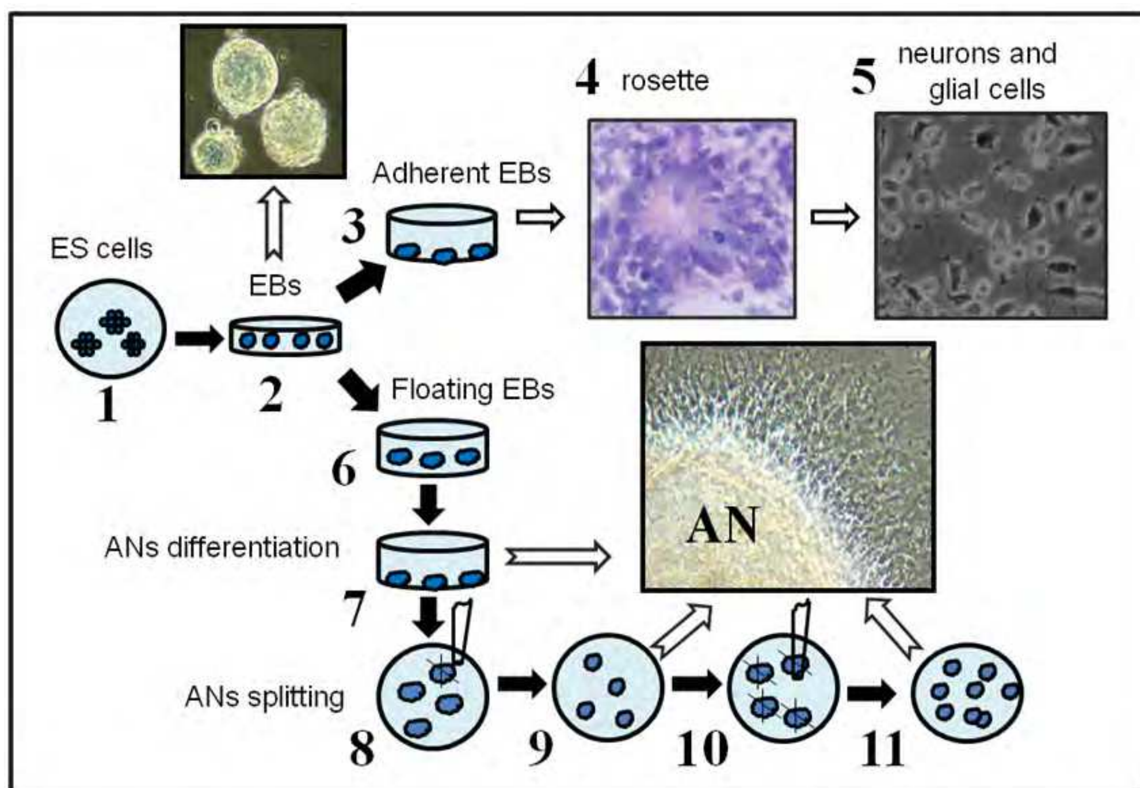

Fig. 4. Differentiation of ES cells towards neurons. 1-2: EBs formation; 3-6: 3-D model; 7-11:2-D model. (1) Pluripotent ES cells in basal culture medium. (2) EBs formation using suspension cell culture or hanging drop protocol. (3-5) Adherence of EBs, in serum-free medium with or without RA, production of neuroepithelial cells, (4) rosette formation, (5) neurons (white-brilliant) and glial cells (black) production. (6-11) culture of floating EBs in serum-free medium in the presence of RA, (7, 9, 10) RA removal, EBs adherence and ANs formation, production of neuronal precursor and mature neurons, (8, 10) ANs mechanical splitting and transfer using glass pipette. White arrows showed in (2) EBs (phase contrast), in (3) rosette (Hematoxylin & Eosin staining) in (5) neurons (phase contrast), in (7, 9, 11) AN with outgrowing neurons (phase contrast).

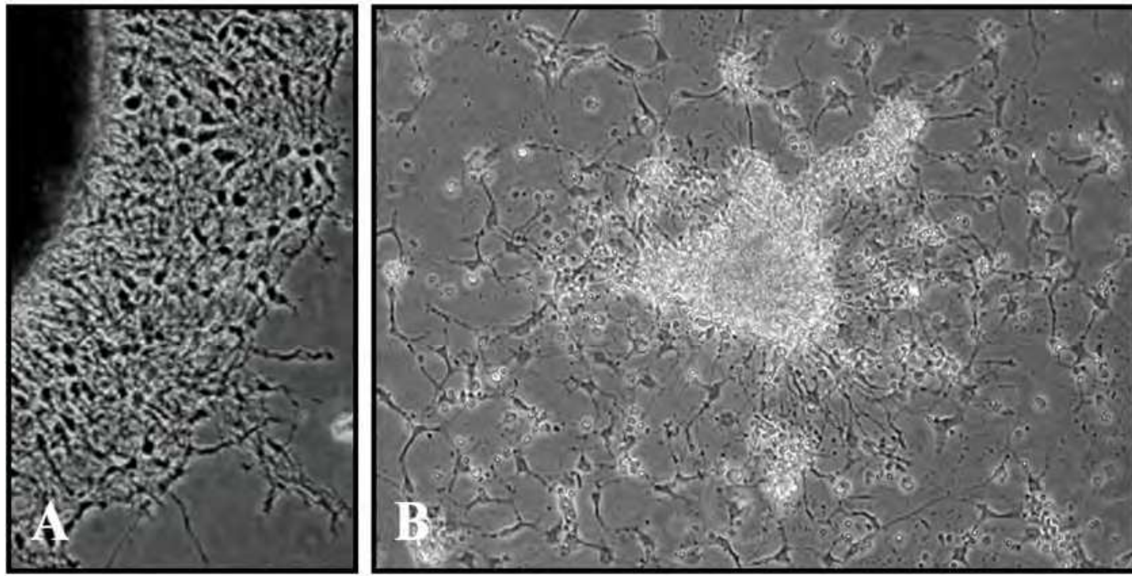

Fig. 5. Differentiation of PSC-derived adherent neurospheres. A) AN (black) producing a network of connected neurons. B) AN (white) after mechanical splitting and transfer. Migration of neurons also can be observed. Light microscopy (phase contrast).

These ANs present expression of the neural progenitor cell markers, such as Sox1 and Nestin just after plating. Following differentiation, the inner part of the AN, continuously expressed Sox 1 and Nestin proteins, while outgrowing neurons, which form an extensive neurite net around the AN expressed beta III-tubulin (neuron-specific marker) (Fig. 6).

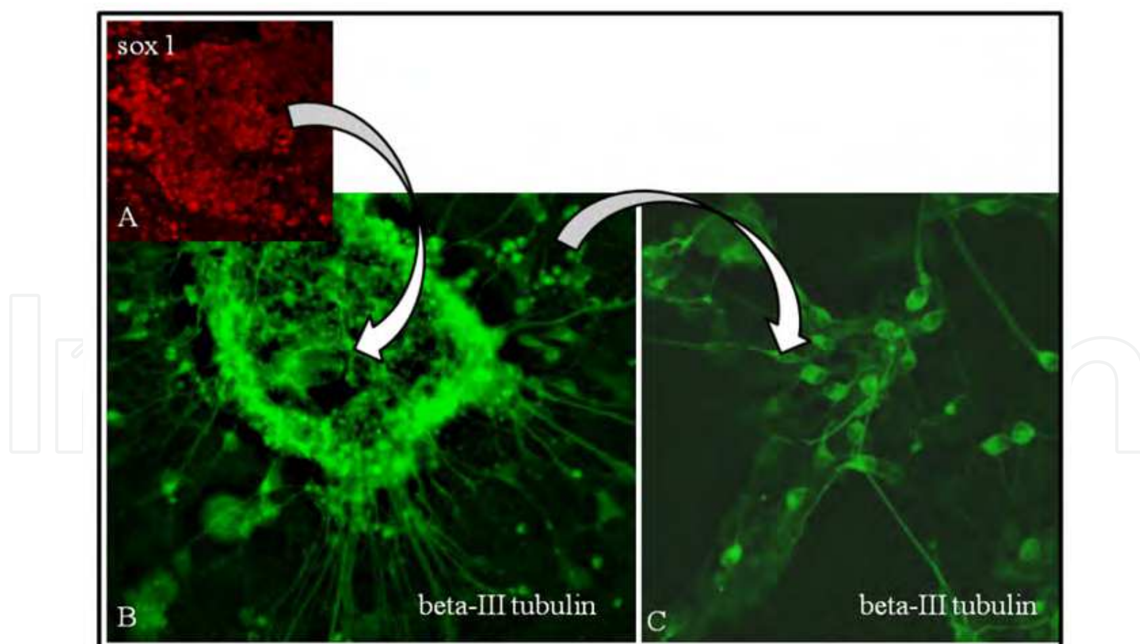

Fig. 6. Expression of neuroepithelial and neuronal markers in AN. A) Expression of Sox 1 protein (red), which express in the nucleus of progenitor cells localized in the inner part of AN. B) Expression of beta-III tubulin protein (green) at the periphery of AN and in outgrowing neurons. C) Higher magnification of extensive neurite net in (B) showing interconnected neurons.

The expression of other neuron-specific proteins, such as MAP2 (microtubule-associated protein 2), NF-M (neurofilament medium protein), Tau (a microtubule-associated protein), NeuN (neuronal nuclei marker), GABA and 5-HT (5-hydroxytryptamin), was observed in neurons derived from ANs each time after splitting and mechanical transfer, which was maintained during three months. Electrophysiological analysis, by using the patch-clamp technique, in long-lasting culture of AN-derived mature neurons, showed the presence of ionic channels and membrane electrical potentials typical of electrically excitable cells, which is a characteristic feature of functional CNS neurons.
This method of mechanical splitting and transfer of ANs is advantageous because it avoids the trauma associated to the trypsin treatments and mechanical dissociation and, so forth, may improve the survival of committed precursors able to differentiate into neurons. It is well-known that CNS precursors are localized in stem cell niche of organisms, which guarantees their continuous growth and renewing, and also the production of differentiated cells. In our model, ANs provide a constant microenvironment (*in vitro* niche) for the neuronal progenitor cells, which can be maintained for at least twelve weeks in culture, following repetitive mechanical splitting and transfer. Since expression of GFAP (Glial fibrillary acidic protein) gene has not been detected, it seems that AN direct the fate of non-committed precursors toward the neurons generation.

2.3.3 Importance of lineage selection for transplantation studies in regenerative medicine

A majority of available protocols for neural differentiation result in the generation of multiple cell types of committed neural precursor to a fully differentiated, post-mitotic neural cell. The selection and expansion of ES-derived neural precursors is a material for transplantation studies focusing on diseases as Parkinson's or Alzheimer's disease, or neural damage following stroke or injury. Such protocol is important due to the elimination of PSCs from the transplanted cell population, which can generate teratocarcinomas (Zhang et al., 1996, Deacon et al., 1998, Bjorklund et al., 2002). Commonly genetic engineering methods are used for lineage selection on differentiating ES cells to purify neural precursors. These techniques rely on the introduction of a reporter/selection cassette into a locus with restricted expression in the desired cell type by homologous recombination. Thus, to address lineage selection of neuronal precursor one copy of the pan-neural gene Sox1 has been replaced by the dual selection/reporter cassette egfpIRESpac in ES cell line, which confers cell-autonomous green fluorescence and puromycin resistance to cells that express Sox1. This gene is not expressed in ES cells. Its expression is limited to neuroectodermal cell, and undifferentiated ES cells are not fluorescent. Upon neural differentiation, Sox1 is activated and the cells produce green fluorescence enabling further purification of both neural and non-neural cells generated during differentiation. Fluorescence-activated cell sorting (FACS) is used for the isolation of both Sox1–GFP-positive and -negative cells allowing further analysis (Li et al., 1998, Pevny et al., 1998, Wood et al., 1999, Ying et al., 2003).
The comparison of AN and the above described techniques demonstrated that we succeeded to establish very simple and long-term protocol for generation of Sox1 positive cells. It is useful to note that 3 months of several mechanical splitting, Sox1-positive cells maintain the expression and continuously produce outgrowing beta III-tubulin-positive cells, while expression of GFAP gene has not been detected (Hayashi et al., 2010). Quantification of precursors and mature neurons demonstrated stable production of both Sox1 and beta III-tubulin proteins during the first 2 months. At the end of the third month, Sox1-nestin-

positive cells were maintained at a similar level as before (~83%), whereas the number of immature neurons (~45%) decreased 1.5-fold, suggesting delay of the maturation process (~32%). Moreover, we showed that under the described conditions, dopaminergic, GABAergic, and serotonergic neurons can be produced. Therefore, generation of 2-D model is of great importance because allows expansion of neural progenitor without genetic modification from primary ES cell culture. Our AN protocol is especially advantageous for the future of regenerative medicine and treatment of neurodegenerative diseases, which will provide more tools for a safety clinical protocol with the advantage of lacking the intermediate effects from non-neural cells.

2.3.4 Importance of microtubule associated proteins for neuronal differentiation

Neuronal migration has been studied extensively in diverse mammalian species and the sequence of events that occurs during cortical development is shared by all mammals. (Gleeson & Walsh 2000, Walsh & Goffinet 2000). During neurogenesis, neural precursors are generated, which proliferate and differentiate into immature postmitotic neurons. These immature cells migrate from the ventricular zone (VZ) to preplate, a layer at the surface of the developing cerebral cortex, splitting the preplate and forming the cortical plate, which further develops into the cortex. Following immature neurons migration from the VZ, cortical lamination is established in an inside-out fashion. In the deep of the cortex, the earliest-born neurons end up, while later-born neurons localize at more superficial layers of the cortex residing near the pial surface. Synaptogenesis and apoptosis of neurons occur at the final stages of cortical development. Indeed, the migration of neurons requires the same steps, which is necessary for migration of any cell type. The signals of environment for attraction and repulsion; the nucleus dislocation from central position to the periphery, a process called nucleokinesis; and a mechanism for migration end up. Microtubule associated proteins (MAPs), for instance, Lis1 and Ndel1, have been shown to be essential for neuronal differentiation and cell migration during the CNS development and also in the adult nervous system.

2.3.4.1 Lis1 and Ndel1

Haploinsufficiency of Lis1 results in lissencephaly, a human neuronal migration disorder (Reiner et al., 1993, Saillour et al., 2009). Patients with type 1 Lissencephaly disorder, have a reduction in brain folding, and aberrant distribution and orientation of neurons in several brain regions. Lis1 binds with high affinity to a protein called Ndel1. Both proteins can complex with cytoplasmic dynein, the retrograde microtubule motor. Lis1 and Ndel1 are proposed to be important for the regulation of dynein-related events in mitosis and migration (Shu et al., 2004, Yamada et al., 2008, Youn et al., 2009, Hippenmeyer et al., 2010, Zyłkiewicz et al., 2011). Thus, PSCs can provide an important model to study migration defects related to MAPs.

Lis1 is a central component of a protein complex, evolutionarily conserved from fungus to human that regulates nuclear migration (Morris, 2000). Lis1 is able to regulate neuronal migration efficiency in a dose-dependent manner (Gambello et al., 2003). Reduced Lis1 activity results in severe defects in the radial migration of multiple types of neurons, including neocortical projection neurons (Tsai et al., 2005).

Ndel1 is important for normal cortical development and it is involved in microtubule organization, nuclear translocation, and neuronal positioning, in concert with various other proteins, including Lis1 (Shu et al., 2004, Youn et al., 2009). Mutations in the mammals Lis1

gene result in neuronal migration defects (Reiner et al., 1993, Youn et al., 2009, Saillour et al., 2009), while knockdown or ablation of cortical Ndel1 function also results in impaired migration of neocortical projection neurons (Sasaki et al., 2005, Youn et al., 2009). Lis1 and Ndel1 co-localize predominantly in the centrosome in early neuroblasts, and later, redistributes to axons during neuronal development (Shu et al., 2004, Guo et al., 2006, Bradshaw et al., 2008, Hayashi et al., 2010). Thus, Lis1 and Ndel1 are essential for normal cortical neuronal migration and neurite outgrowth.

Currently, Lis1 and Ndel1 were shown to have additional, important functions in the cytoplasmic dynein pathway. They participate in nuclear and centrosomal transport in migrating neurons (Shu et al., 2004, Tsai et al., 2005). Additionally, they influence a centrosome positioning in migrating non-neuronal cells (Dujardin et al., 2003, Stehman et al., 2007, Shen et al., 2008) as well as chromosome alignment, and mitotic spindle orientation (Faulkner et al., 2000, Siller et al., 2005, Liang et al., 2007, Stehman et al., 2007, Vergnolle & Taylor 2007). McKenney and co-authors (2010), using biochemical and biophysical approaches, investigated whether and how Lis1 and NudE (Ndel1) affect dynein motor activity. Results obtained in this work apparently explain the requirement for Lis1 and NudE in the transport of nuclei, centrosomes, chromosomes, and the microtubule cytoskeleton. Additionally, they provide new insight into the molecular basis for lissencephaly, and the mechanism of action of these proteins in a broad range of biological functions.

2.3.4.2 Expression of Lis1 and Ndel1 in ANs

Regardless of the relevance of these proteins for neuronal differentiation, their expression during PSCs differentiation is not well explored yet. Our group was the first to analyze intracellular localization of both proteins in mES cells, undifferentiated and during *in vitro* neural differentiation (Hayashi et al., 2010). The expression of both Lis1 and Ndel1 proteins was observed in undifferentiated cells, which presented co-localization within the perinuclear region (Fig. 7). At early stages of differentiation, just after formation of ANs, Lis1 expression was observed in the cytoplasm, while Ndel1 was in the perinuclear region of committed cells. Following differentiation, when ANs grow in size, the expression of both proteins was no more observed in the area of committed cells. Both Lis1 and Ndel1 proteins were visualized in outgrowing neuritis. Additionally, they co-localized with Tau, which is a marker of MAPs, involved in the microtubule assembly and stabilization. In the same way, Ndel1 and MAP2 were also co-localized. In non-rosette MAP2 positive neurons, Lis1 and Ndel1 proteins co-localized in neuronal cell body and growing axons (Fig. 7).

In attempt to mimic the development of cortical layers *in vitro* and to study the cell migration during the differentiation process, which can be assessed by the analysis of the expression pattern of these proteins, the ANs were allowed to grow for 15 days without splitting. Significant variation in spatial distribution of Lis1 and Ndel1 proteins were observed within 2-D ANs. The expression of Lis1 was observed in the inner part of ANs, in the cells presenting rosette morphology. Unexpectedly, Ndel1 was not expressed in rosette forming cells. Both proteins were co-localized in the cytoplasm of the cells showing neuroblast-like morphology, which were found close to the periphery of AN. Lis1 protein was expressing in the cells very closely localized to Ndel1 expressing cells, which, in turn, were close to the region of outgrowing neurons. Co-localization of Lis1 and Ndel1 expression was detected in cells from upper layer of ANs. Ndel1 was found to interact with centrosomes, suggesting that these cells are early neuroblasts (Fig. 8).

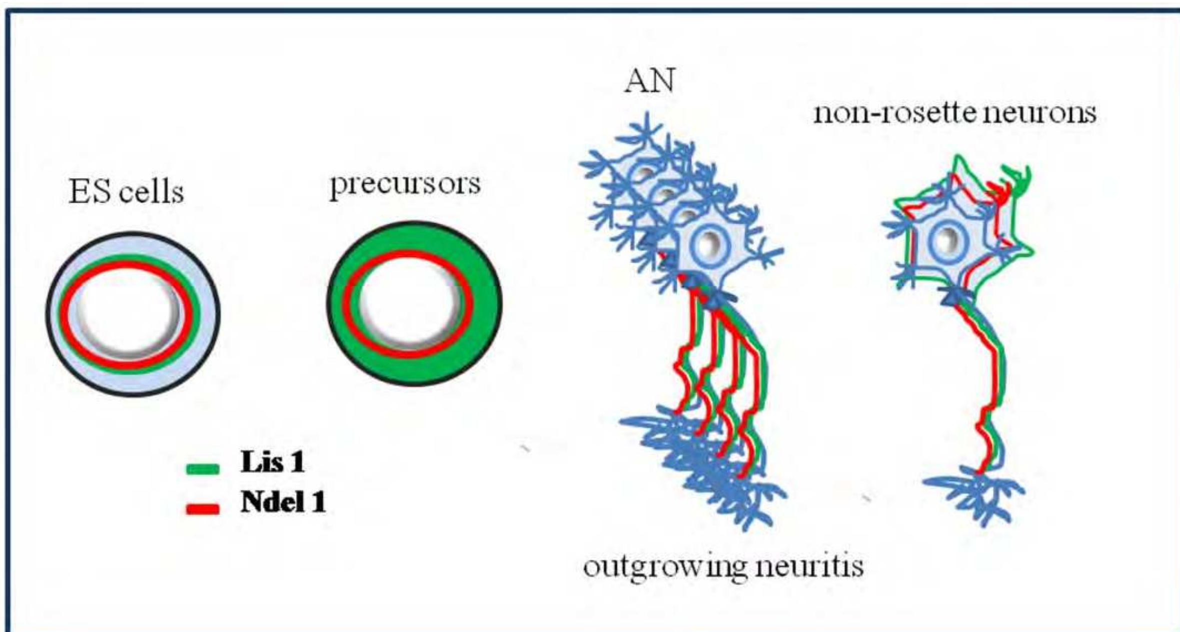

Fig. 7. Schematic presentation of expression pattern of Lis1 and Ndel1 during neuronal differentiation of PSCs. In undifferentiated ES cells, both Lis1 and Ndel1 show a perinuclear co-localization. In neuronal precursors, Lis1 presents a cytoplasmatic and Ndel1 a perinuclear localization. In neurons, at the periphery of ANs, both Lis1 and Ndel1 co-localize in the outgrowing neurites. In non-rosette neurons, these proteins co-localize in neuronal body and neurites.

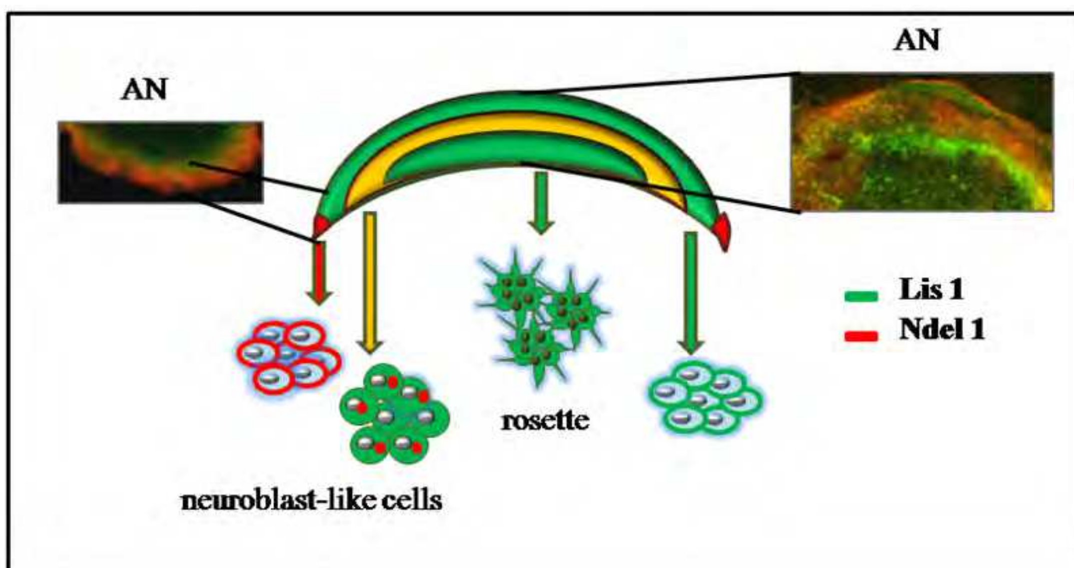

Fig. 8. Schematic presentation of Lis1 and Ndel1 expression in different layers of AN. Lis1 (green) expression was observed in the inner part of AN in the cells organized in rosettes. Intermediate layer (yellow) is composed by the neuroblast-like cells, which express both proteins, Lis1 (green) in the cytoplasm and Ndel1 in centriolos (red) and less in cytoplasm. Upper layer is composed mainly by Lis1 and at the periphery of AN by Ndel1 expressing cells. Proteins expression is also demonstrated by immunofluorescence within AN located on both sides of schematic presentation (Confocal microscopy + Fluorescence).

Interaction of Lis1 and Ndel1 with other cytosolic proteins had been well studied using cultured non-neuronal and/or neuronal cells and the expression of both Ndel1 and Lis1 genes in early neuroblasts derived from embryonic and adult tissues was observed (Sasaki et al., 2000, Shu et al., 2004). Our data demonstrated an expression of only Ndel1 without Lis1 expression in the centrosome region in neuroblast-like cells within differentiated AN. Variation of spatial distribution of Lis1 and Ndel proteins expression was also observed. The ES cells isolated from, for instance, Lis1 or Ndel1 knockout mice, followed by their differentiation into neuronal cells using the present protocol, will permit the elucidation of the real role of each protein during the neuronal differentiation process. Our data suggest that further analysis involving other important MAPs are necessary to allow a better comprehension of the migration mechanism(s) and of the specification fate of neuronal cells during differentiation.

3. Conclusions

PSCs have the capacity to differentiate *in vitro* into neuronal cells spontaneously through EBs formation or in monolayer culture. EBs 3-D model is shown to be more efficient model, which can be improved using serum-free culture conditions and inductors of differentiation (e.g. RA). Following this protocol, neuroepithelial cells could be obtained, which formed rosettes. Further selection and enrichment protocols are needed to isolate culture of committed neuronal precursor, neurons and/or glial cells. This 3-D model provides short-term culture of neuronal cells, which did not allow analysis of neurons migration and survival. It is of note that these AN can be maintained even in the absence of growth factors, without lacking the capacity to produce functional neurons.

Our study demonstrated that ANs is a long-term protocol, which can be used to analyze the process of neuronal differentiation in dynamics. Plating of intact ANs also provides a window of time to the precursor cells for establishing their fate in a 2-D environment. ANs model avoid a stage of rosettes formation directly producing committed progenitors and non-rosette neurons, mimicking process of differentiation of neurospheres form CNS. Mature neurons, obtained from ANs, display ionic channels and membrane electrical potential, which are typical of electrically excitable cells and are also a characteristic feature of functional CNS neurons.

Following mechanical splitting and transfer, these ANs grow continuously, confirming their auto-renewing properties similarly to progenitors of CNS. When maintained untouched during prolonged period (at least 15 days), progenitors inside growing ANs undergo further cell specification. As we demonstrated by the analyses of expression of Lis1 and Ndel1 proteins, both presented differential spatial distribution within the ANs. The discrepancy between patterns of expression of these proteins in neuroblasts isolated from embryonic or adult mouse neuronal tissues, and in those AN-derived cells was observed. AN-derived neuroblasts demonstrated only Ndel1 location in centrosome region, instead of showing the location of both proteins in this region. This indicates that miss expression of proteins, which are responsible for neuronal cells division and migration, can occur during *in vitro* differentiation.

Thus, our protocol provides an efficient experimental model for studying neuronal *in vitro* differentiation mimicking early development, as well as it represents a novel source of functional cells that can be used as tools for testing the effects of drugs on functional neuronal cells.

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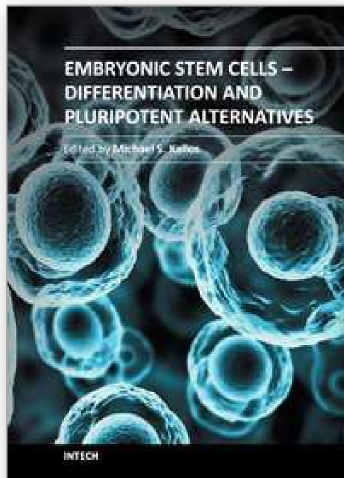

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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