NEURONAL PRODUCTION *IN VITRO* FROM EMBRYONIC STEM CELLS

*Evguenia Bekman, Claudia Valente de Castro, Rita Fior and Domingos Henrique**

Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal

ABSTRACT

Cell therapy in the nervous system is a promising strategy to cure diseases like Parkinson's or for nerve regeneration in spinal cord lesions. However, it requires the *ex-vivo* generation of neurons or their immediate progenitors in sufficient numbers, and of the correct neuronal type, which can then be used for transplantation. To achieve this, an efficient method for the *in vitro* production of neurons was established, starting with Embryonic Stem (ES) cells. We show that this method mimics several steps of the neurogenesis process in the developing embryo, with cultured cells being able to organize in 3D structures that resemble embryonic neural tubes. This method might prove to be extremely useful to generate differentiated neurons for future transplantation studies in the mammalian brain.

INTRODUCTION

One of the most puzzling natural phenomena is the creation of a complex multicellular organism from a single totipotent cell, the zygote. For many decades, the process of embryogenesis has been a subject of intense research. The accumulated knowledge has allowed the recent emergence of several strategies to[**] achieve the *in vitro* production of differentiated cells, tissues and organs, for therapeutic purposes[1]. In this new field, named Regenerative Medicine, a particular type of cell – stem cell, has a fundamental role. Stem

cells are characterized by their ability to self-renew and to generate [2]differentiated progeny, being able to functionally reconstitute a given tissue *in vivo*.

Throughout embryonic development, several stem cells emerge that differ in their differentiation potential. They can be found in embryonic tissues, fetal tissues (e.g. Embryonic Germ (EG) cells, fetal multipotent cells) and adult tissues (e.g. Hematopoietic Stem Cells – HSCs). An important discovery involved the characterization of the so-called Embryonic Stem (ES) cells, which were first isolated from mouse blastocysts[2,3], one of the earliest stages of embryonic development. These cells are pluripotent, i.e., able to differentiate into cells from the three germ layers, and can be cultured for long periods of time without loosing this ability. In contrast, adult stem cells are more limited in their potential and can be found in specialized tissues in the adult organism, such as the brain, being able to self-renew and differentiate only into cells from the originating tissue.

Given their exclusive properties, stem cells are promising candidates for tissue engineering, cellular therapies and drug screening[4]. The *in vitro* reconstitution of neurogenesis, involving the production of neuronal precursors and/or differentiated neuronal subtypes, is one of the most sought-for processes. Successful attempts have been made to achieve in vitro neuronal differentiation from ES cells, either by embryoid body (EB) formation in the presence of retinoic acid[5], by co-culture with stroma/conditioned medium[6,7], or by monolayer differentiation[8]. However, as ES cells are pluripotential and readily differentiate into almost any cell type, lineage selection is usually essential to ensure homogeneity of the differentiated population[9]. Neuronal differentiation from neural stem cells (NSCs), either adult or embryonic, has also been achieved and later tested in transplantation studies[10]. However, clonal propagation of neural-stem (NS)-derived precursors is limited and a switch from neuronal to mostly glial fate occurs during prolonged culture of these cells.

A successful strategy to achieve production of neuronal precursors *in vitro* must take into account what is known about neurogenesis in the vertebrate embryo and the regulatory events involved in the process. Neural induction happens early during embryonic life and involves both FGF signaling and the inhibition of BMP signaling by SMAD1 phosphorylation[11,12]. Neurogenesis begins when ectoderm cells receive these induction signals coming from the underlying notochord, forming a new embryonic tissue, the neuroepithelium, a thickened epithelial sheet where cells form a tightly-packed monolayer with constricted apical surfaces and elongated fusiform cell bodies. Within the neuroepithelium, cells start to express *Sox1*, a Sry-related transcription factor specific to early commitment stage of neurogenesis[13]. Neuroepithelium then folds into a tube-like structure, the neural tube, where the concerted action of anterio-posterior and dorso-ventral patterning processes leads to the regionalization of the major subunits of neural tube, such as forebrain, midbrain, hindbrain and spinal cord, and their subsequent subdivision.

Neuroepithelial cells show a marked apico-basal polarity, which has both structural and functional importance (Fig. 1). The apical domain is located at the luminal surface and is delineated by the presence of apical protein complexes, like the PAR polarity complex[14], as well as by the presence of junctional structures where N-cadherin and β-catenin accumulate[15]. Centrosomes also localize apically in neuroepithelial cells, which enter mitosis close to apical surface due to the characteristic interkinetic nuclear movement[16]. This particular organization of neural tube is important for the coordinated production of neurons and glia. Neighboring neuroepithelial cells signal to each other through the interaction of the Delta ligand and the Notch receptor, resulting in the inhibition of differentiation of the cells adjacent to newborn neurons, which will later accumulate at the basal portion of the neural tube and migrate to dorsal root ganglia[17]. This process of lateral inhibition, mediated by the Notch pathway, is responsible for the maintenance of neural progenitors throughout the process of neurogenesis and, consequently, for the timely production of the right number of neurons at each time of embryonic development[18].

MATERIALS AND METHODS

The 46C ES line[8] was used along this work and was kindly provided by Dr. Austin Smith (Edinburgh University, U.K.). It contains the coding sequence of GFP inserted in the *sox1* gene and has been used successfully to follow neural commitment[8]. N2B27 and RHB culture media were obtained from StemCellSciences Co. (U.K.). FGF-2 was obtained from Peprotech. Antibodies were obtained from Upstate Biotech. and Santa Cruz Biotech. (USA). Immunofluorescence studies were performed as described[19].

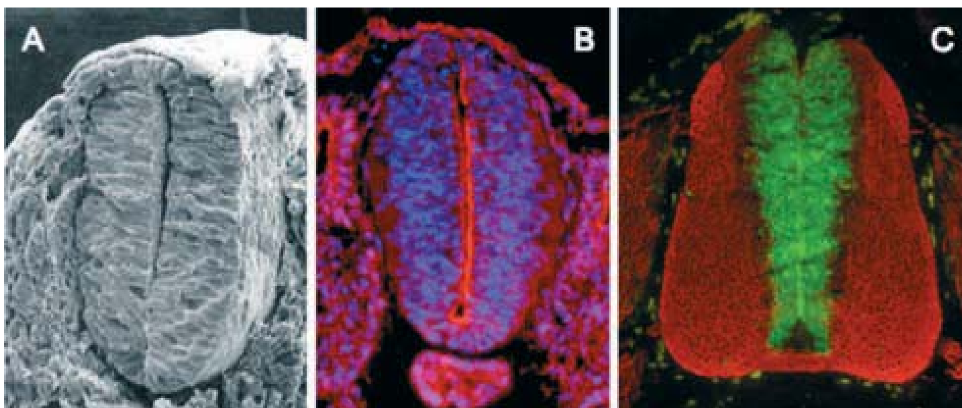

Fig. 1 – **A**, Scanning electron microscope image of the transverse section through the E9 mouse embryo, revealing the closed neural tube. B and C: E2 and E3 chick neural tube, respectively. **B**, β-catenin staining in red, nuclei in blue. **C**, *Hes5-3 in situ* hybridization (green), delimiting progenitor zone, and Tuj1 immunostaining (red) for nascent neurons located more basally in the neural tube.

RESULTS AND DISCUSSION

The simplest way to reconstitute neural commitment *in vitro,* and achieve efficient neuronal production, relies upon monolayer differentiation of ES cells, a method developed by Ying and co-workers[8]. In this method, ES cells are cultured in defined medium which does not contain serum and is thus free from BMP-imposed inhibition of neural fate. Prior to initiate neural differentiation, cells are grown overnight in a dense culture, allowing establishment of multiple intercellular contacts. These dense cultures are then replated at low density in defined serum-free medium (N2B27), which contains N2 supplement (insulin, apo-transferrin, sodium selenite, progesterone and putrescine), and B27 supplement, containing retinoic acid. Though none of these components, with the exception of apo-transferrin, is essential for neuronal commitment[20], their combinatorial effect results in up to 90% neuronal commitment by the sixth day of continuous culture, as measured by the activity of the Sox1-GFP knock-in allele present in the 46C ES cell line (Fig. 2).

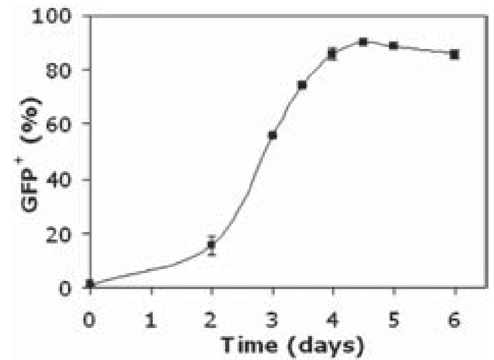

Fig. 2 – Percentage of GFP-expressing cells during monolayer differentiation. The use of this specific cell line allows FACS-based monitoring of the dynamics of neuronal commitment.

Most interesting, by day 5-6 in monolayer culture, the commited neural progenitors form either rounded clusters of GFP-positive cells, or extended sheets with patchy GFP distribution. Immunostaining for apical markers, like the zona occludens protein ZO-1, reveals that GFP-positive patches of cells are organized in rosette-like structures resembling small neural tubes, with well-defined apical domains, around which GFP-positive neural progenitors are organized (Fig. 3).

This suggests that neuroepithelial cells are able to achieve a correct apical polarity in the monolayer differentiation condi-

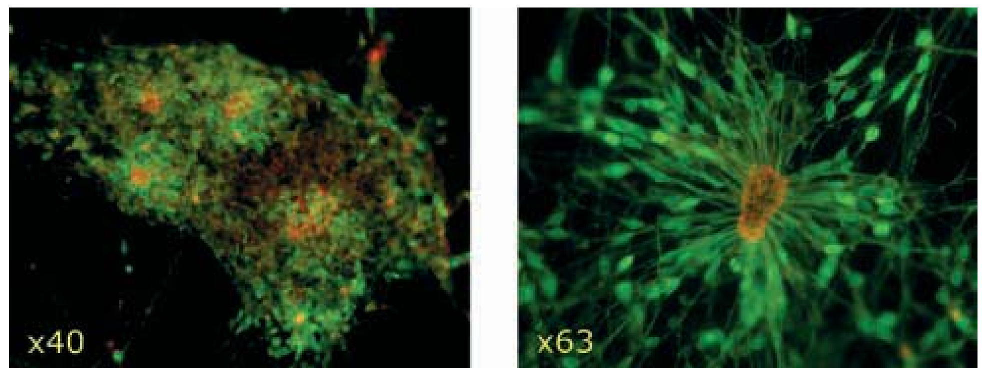

Fig. 3 – **A**, An example of day 6-monolayer culture of 46C cells, stained with ZO-1 antibody (red), and GFP (green). **B**, Rosette structure of neural precursors, formed after replating of day 6-monolayer culture onto poly-D-lysine/laminin-coated dish.

tions employed in these experiments. To confirm this, we have performed a detailed characterization of the rosette-like structures obtained during the monoloayer differentiation protocol, using immunofluorescence localization of several known apical proteins. As shown in Figure 4, neuroepithelial cells within rosettes have a polarized distribution of junctional components like N-cadherin and β-catenin, which appear to localize close to the luminal region of such rosettes. The PAR polarity complex is also localized at the same luminal region, confirming that this region constitute the apical domain of rosette's neuroepithelial cells. This is also confirmed by the localization of centrosomes at the region below the apical domain, and by the localized occurrence of mitotic figures in the same region, as it normally happens in the embryonic neural tube. Concurrently, differentiated neurons, detected by the Tuj1 and HU antibodies, are present outside the rosette strutures, mimicking their normal migration from the neural tube ventricular region.

In summary, the monolayer differentiation method constitutes an excellent approach to study neurogenesis *in vitro*, as it permits to reconstruct, at least partially, the tridimensional organization of embryonic neural tube. We have found that several independent ES cell lines show a similar behaviour during *in vitro* differentiation, indicating that this processes is universal and must be important to achieve normal neuronal commitment and differentiation. Furthermore, as neural progenitors can be found only within the rosette structures, while neurons migrate out of these structures, we propose that a proper epithelial organization is important for neuronal commitment in vitro, as well as to achieve an efficient neuronal production.

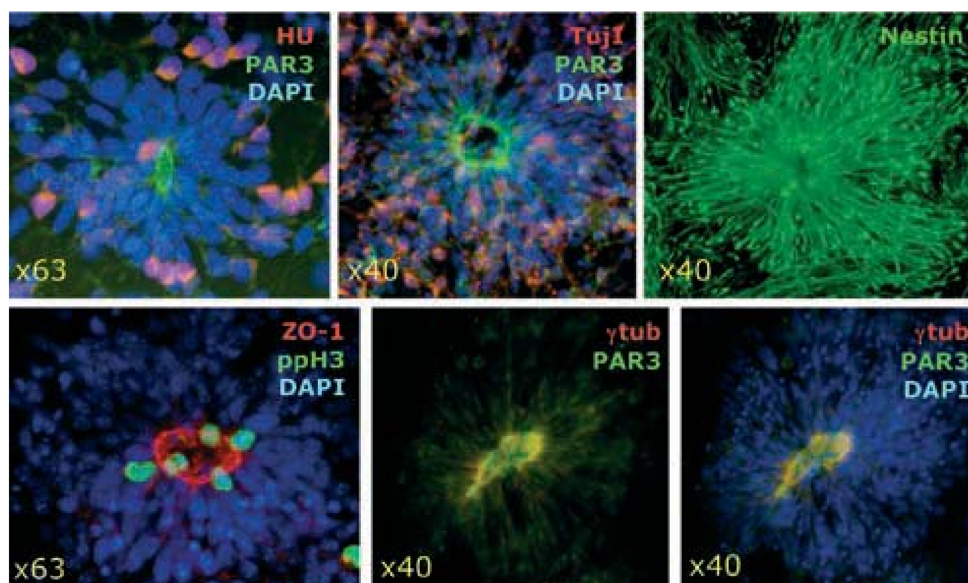

Fig. 4 – Neural tube-like structures formed by S25 ES cells after 12 days in monolayer culture. After the initial 6 day-monolayer culture, cells were replated on poly-D-lysine/laminin coated coverslips and cultured in the same conditions for 6 days more, then fixed and stained with indicated antibodies. S25 ES cell line bears a recombinant Sox2-βgeo allele and shows the same efficiency of neural commitment in monolayer culture as 46C line, as estimated by Nestin immunofluorescence.

* Corresponding author (henrique@fm.ul.pt)

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