Design and operation of bioreactor systems for the expansion of pluripotent stem cell-derived neural stem cells

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Abstract— **Neurodegenerative disorders, such as Parkinson's disease, Huntington's disease, and multiple sclerosis, affect millions of people worldwide, with devastating impact on the patients and families. A reliable method to obtain cells for replacement therapy, using *ex-vivo* culture techniques, would be of great value for the treatment of these conditions. This project focuses on the development of bioreactor culture systems for the large-scale expansion of neural stem cells, starting with a model line of mouse embryonic stem cell-derived neural stem cells under adherent culture conditions. Adherent conditions are an alternative to conventional culture of NS cells as aggregates and may circumvent problems associated with this system. Small scale stirred bioreactors were successfully used for mNS cell expansion, with microcarriers to support cell adhesion and proliferation, with retention of neural stem/progenitor cell markers. The system was optimized by determining the best values for parameters like stirring speed, microcarrier concentration or feeding regimen. The system here described may open a new door for applications requiring high numbers of mouse neural cells, providing an efficient way for their generation. Furthermore, the know-how obtained with this work may be applied for the development of an equivalent system for human cells, which may find clinical applications.**

Index Terms—neural stem cells; expansion; bioreactors

I. CONTEXT (HEADING 1)

Neural stem (NS) cells are self-renewing multipotent cells present in the developing and adult mammalian CNS [1]. They generate the neurons and glia (astrocytes and oligodendrocytes) of the developing brain and also account for the limited regenerative potential of the adult brain.

Different protocols for NS cell expansion have been developed in both floating and adherent conditions. The propagation of floating cell clusters, called "neurospheres", in the presence of fibroblast growth factor 2 (FGF-2) and epidermal growth factor (EGF), is widely used for NS cell expansion *in vitro* [2, 3]. Neurospheres contain NS cells and progenitors mixed with differentiated cells embedded in a complex extracellular matrix and cells expanded *in vitro* with this strategy can still be operationally characterized by self-renewal and multipotential differentiation.

However, NS cell expansion as neurospheres shows severe limitations for cell culture like heterogeneity within and between neurospheres, oxygen and nutrients diffusion limitations and waste accumulation in the cluster centre, stem cells maintained within neurospheres have an uncertain relationship to CNS precursors *in vivo*, and neurospheres tend to differentiate much more readily into astrocytes than neurons *in vivo* [4]. The expansion of NS cells on adherent conditions may circumvent most of these limitations.

Different sources of NS cells are available, such as embryonic stem (ES) cells, that can give origin to NS cells by *in vitro* differentiation, the fetal brain or the adult brain, and thus determining the best sources for the *in vitro* derivation of NS cells and the optimization of protocols for their stable and clonal proliferation are central challenges of stem cell research [1] .

ES cells are derived from the inner cell mass of the blastocyst [5, 6] and have unlimited self-renewal capacity and multilineage differentiation potential into any cell type [7]. The generation of homogeneous populations of NS cells from ES cells and their further differentiation into mature neural phenotypes allows their potential use for treatment of neurodegenerative diseases, neural drugs screening and also gene therapy settings. Although it is anticipated that a large number of cells will be required for those applications, the large-scale expansion and controlled differentiation of tissue-specific stem cells on bioreactors presents major biological and technological hurdles to be overcame.

Stem cells from different origins are currently cultured under static conditions on tissue culture (TC) plastic either as suspension cells/aggregates or adherently. However, despite their widespread usage, these culture systems are not amenable for large-scale applications since they are limited in their productivity by the number of cells that can be supported by a given surface area/volume. In addition, these systems have serious limitations such as a non-homogeneous nature, resulting in concentration gradients (growth factors, metabolites, pH, dissolved oxygen…), and difficult or even impossible on-line monitoring and control [8].

II. GOALS

This project focuses on the development and operation of bioreactor culture systems for the large-scale *ex-vivo* expansion of stem cells under adherent culture conditions. Mouse embryonic stem (mES) cell-derived neural stem (NS) cells are used as model.

Mouse NS cells were already cultured as neurospheres on bioreactors [2]. However, as described above, these culture conditions potentially induce stem cells to differentiate and thus may not be optimal to maintain their multipotency. Furthermore, novel adherent culture systems have been established for the expansion/differentiation of stem cells in a homogeneous way, which were found to be more efficient and easier to control when compared with aggregates [4].

The stirred suspension bioreactor, very well characterized for both microbial and mammalian cell cultures, can be an alternative system for neural stem cell culture. This type of bioreactor can provide a homogeneous environment, easy sampling for data collection, and control of medium composition [8]. In this project, stirred suspension bioreactors are evaluated using microcarriers to support cell adhesion and proliferation and/or differentiation. Initially, small scale spinner-flasks are evaluated but the protocols developed with this system are meant to be translated and adapted to larger scale, fully-controlled bioreactors.

III. Team and Institutions

The research team for this project, developed at the Centre of Biological and Chemical Engineering, Insitute for Biotechnology and Bioengineering - Instituto Superior Técnico is leaded by Professor Joaquim M.S. Cabral and includes Carlos A.V. Rodrigues, Maria Margarida Diogo, Cláudia Lobato da Silva and.

IV. Results

The first steps of this project consisted in the characterization and optimization of the culture of a mES cell-derived NS cell line under static conditions [9]. Particular attention was given to the influence of oxygen in cell proliferation. Hypoxic conditions (culture under a 2% oxygen atmosphere) were found to lead to higher growth rates without negatively affecting cell potential [9].

As mentioned before, static culture in tissue culture flasks or similar systems show various limitations, specially in terms of scale up, and thus effort was made to implement dynamic culture of the mNS cell line used before. Culture of NS cell in spinner-flasks, under adherent conditions, requires a support where cells can attach and in this work microcarriers were used for that purpose. The first step for establishing a culture protocol was a screening of different commercially available microcarriers. Since this project aims the elimination of animal-derived products form the culture, only xeno-free microcarriers were tested, as well as a serum-free culture medium. Different core materials and surface coatings were evaluated, initially under static conditions, being the best results obtained with spherical crosslinked polystyrene beads coated with an extracellular matrix protein or with silica glass. These microcarriers were then tested under dynamic conditions, in the spinner-flasks. Superior performance was observed with extracellular matrix protein coated-microcarriers. Importantly, high cellular viability and the expression of nestin, a marker of neural stem/progenitor cells, was retained when cells were cultured for up to 9 days on the microcarriers, in the spinner-flask (figure 1), confirming that these culture conditions, in particular the shear stress caused by agitation, is not detrimental for the cells.

Initial cell attachment to the beads is a key step for the culture success and therefore different inoculation strategies were compared. Intermittent stirring was shown to be less efficient than constant stirring, probably because with the latter approach cell clumping was minimized, favouring an homogeneous attachment to the beads.

The subsequent steps were the optimization of culture parameters, such as stirring speed, culture medium feeding and microcarrier concentration. A stirring speed of 60 rpm was found to be optimal. While lower speeds lead to inefficient microcarrier suspension and mixing as well as lower performance in terms of surface aeration, higher speeds probably lead to excessive shear stress (figure 2). After 6 days, a 40-fold increase in cell number was observed, with cell viability above 95%.

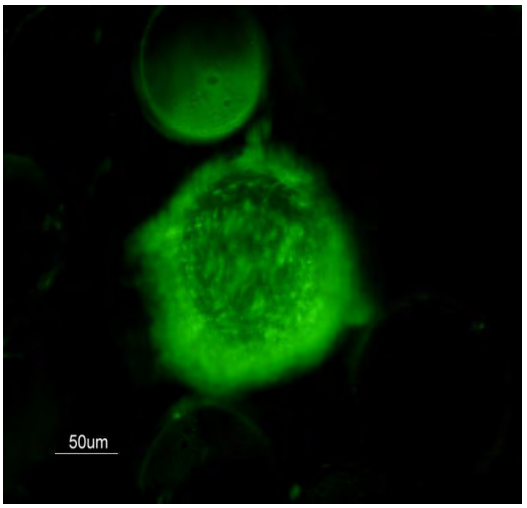

Figure 1- mES cell-derived NS cells cultured on microcarriers express Nestin, a protein expressed by neural stem/progenitor cells

Microcarrier concentration is also expected to have an important role as a higher number of microcarriers provide a larger area for cell attachment and growth and facilitate initial cell-bead contacts but excessive concentrations may lead to bead-bead collisions that may cause damage to the cells. Finally, culture medium feeding is also a critical parameter. The renewal of culture medium has mainly two important roles: replenishment of nutrients, like glucose or glutamine and removal of harmful metabolites, like lactate. Thus, the metabolic profile of the culture, in terms of glucose, glutamine and lactate concentrations over time, was determined in order to avoid nutrient scarcity or metabolite accumulation. In the case of NS cells, the frequency of growth factors supply was also found to be crucial.

The validation of this bioprocess for mES cell-derived NS cell expansion will be concluded after the demonstration of the multipotential differentiation of the spinner-flask expanded cells into neuronal and glial cells.

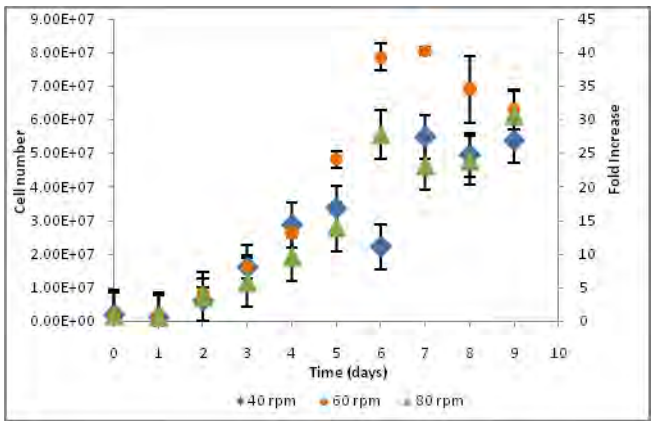

Figure 2-Effect of stirring speed on mES cell-derived NS cells. Fold increase in cell number, shown in the secondary axis, corresponds to the ratio between cell number in a particular time and initial cell number.

V. Conclusions and Planned Developments

Large scale expansion of NS cells capable of multipotential differentiation into neural cells is expected to be a key step for diverse applications, including screening of new drugs, fundamental studies of stem cell biology and also for regenerative medicine. The project here described intends to address this challenge, with the application of bioengineering principles for the development of reproducible mNS cell expansion protocols, in fully-controlled bioreactors and in animal product-free conditions. The results obtained so far show the feasibility of adherent culture of mNS cells in small scale stirred reactors - spinner flasks - using spherical microcarriers as support for cell growth. Although the existing results are still in small scale, scale-up to larger scale, computer-controlled reactors is anticipated to be straightforward and may lead to process optimization in terms of parameters not studied yet, like oxygen tension. Additionally, different bioreactor configurations may be tested, being of particular interest those where mixing and mass transfer is achieved without an impeller.

The work developed with mouse cells may provide an important tool for applications where high numbers of mNS cell or mNS cell-derived cells are required. Integrated NS cell expansion and differentiation, for instance into dopaminergic neurons or oligodendrocytes would be a further enhancement of the process, with outstanding potential. Beyond these applications, the results obtained may constitute the basis for the design of a similar system for human NS cells. Human NS cells, if cultured under xeno-free conditions and complying with GMP regulations, may find application in clinical settings, for instance in the treatment of neurodegenerative diseases or pharmacological studies.

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References

[1] Conti L, Cattaneo E. Neural stem cell systems: physiological players or in vitro entities? Nat Rev Neurosci. 2010;11:176-187.

[2] Kallos MS, Sen A, Behie LA. Large-scale expansion of mammalian neural stem cells: a review. Med Biol Eng Comput. 2003;41:271-282.

[3] Reynolds BA, Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 1992;255:1707-1710.

[4] Conti L, Pollard SM, Gorba T, et al. Niche-Independent Symmetrical Self-Renewal of a Mammalian Tissue Stem Cell. PLoS Biology. 2005;3:e283.

[5] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981;292:154-156.

[6] Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998;282:1145-1147.

[7] Smith AG. Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol. 2001;17:435-462.

[8] Cabrita GJ, Ferreira BS, da Silva CL, et al. Hematopoietic stem cells: from the bone to the bioreactor. Trends Biotechnol. 2003;21:233-240.

[9] Rodrigues CA, Diogo MM, da Silva CL, et al. Hypoxia enhances proliferation of mouse embryonic stem cell-derived neural stem cells. Biotechnol Bioeng. 2010;106:260-270.