

Scientific Experiences Report

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Experiences, procedures and projects during the stay at the cooperation partner:

The stay at the cooperation partner in Lund (Stem Cell Center Lund, Sweden; Section of Restorative Neurology, Head: Prof. Zaal Kokaia) gave me the opportunity to learn several techniques and methods about preparation and cultivation of stem cells of certain origin as well as their transplantation. Within a project I was able to take part on animal behavior tests, perfusion, immunohistochemistry and analysis of the results.

Therefore the following explanations will be divided into three parts: (I) preparation and cultivation of stem cells of different origin, (II) transplantation procedures and (III) insights into the cooperation project.

I. Preparation and cultivation of stem cells of different origin

Neural stem cells are defined as cells that either gives rise to or derives from the cells of the central nervous system and have the unique properties of stem cells, i.e. self-renewal and multipotentiality. One of the widely used methods of expanding neural stem cells under culture conditions is based on the capacity of these cells to divide continuously when cultured in serum-free medium supplemented with various growth factors. One common method used is to grow neural stem cells as free-floating aggregates of cells called neurospheres. Neurospheres can be generated from several structures of the embryonic and adult mammalian brain.

To obtain neural stem cells from rat and mouse medial (MGE, primordial globus pallius) and lateral (LGE, primordial striatum) ganglionic eminences a female mouse or rat at embryonic age 13.5 or 15.5 days is needed. After sacrificing the animal all embryos will be transferred to a Petri dish with L-15 medium. Under a dissection microscope the embryo is decapitated by cutting the plane between eyes and nose. After clearing the brain of skull and meninges a longitudinal incision close to the fissure on one of the hemispheres is made and the tissue need to be folded in order to expose the ganglionic eminences. To separate LGE and MGE properly first a cut between both structures need to be done. To remove the LGE the structure need to be cut along the bottom and is then transferred to a tube with fresh L-15 medium. The MGE can be dissected by cutting below the structure and picking it straight up. It can then be transferred to L-15 medium, too. The same procedure is done on the second hemisphere. Each individual structure can be cultured or they can be pooled to get more cells.

After the collecting the L-15 medium is removed and replaced with 200 μ l DMEM/F12 basic medium. After 15min incubation (humidified incubator, 5% CO₂, 37°C) the suspension will be triturated 10-30 times until a single cell suspension is made. Cells are counted and plated on uncoated flasks at a density of 10-50 cells/ μ l in DMEM/F-12 medium supplemented with 20ng/ml EGF and 10ng/ml FGF.

Cells are cultured in a humidified incubator at 37°C with 5% CO₂. Cells are fed by adding 0.5 -2ml fresh DMEM/F-12 medium supplemented with 20ng/ml EGF and 10ng/ml FGF twice a week.

Cells are passaged when spheres are around 200-300 μ m in size but before the inner core of the sphere becomes non-transparent. For the passage cells are collected and centrifuged at 1,000rpm for 5min. The supernatant is removed and 1ml of basic medium is added. The suspension is triturated 20-30 times with a 1ml pipette, then a 200 μ l pipette tip is attached to the 1ml tip and triturated another 20-30 times. The suspension is passed through a 40 μ m cell strainer and the cells will be counted. The cells are replated in basic medium with growth factors as before.

On the day of transplantation, the neurospheres are passed through a 100 μ m cells strainer to avoid clumping of the cells in the Hamilton syringe during transplantation. Cells are collected by centrifugation (1,000rpm, 5min) and resuspended in HBSS. The cells are counted and resuspended to a final concentration of 100,000 cells/ μ l. During the transplantation the cells are kept on ice and aren't used longer than 5 hours in HBBS. After the transplantation session, the neurosphere suspension is replated in order to assess cell viability.

For exercising of the transplantation and for the cooperation project humaniPS-NS Rosette cells (by Phillip Koch/ Oliver Brustle) were used. This hiPS NS cells are a rosette type of self-renewing human ES cell-derived neural stem cells, which have a potential for in vitro instruction and synaptic integration.

This cells are growing as monolayers and therefore need coated flasks. For coating a flask is first incubated with Poly-L-Ornithin (1.5mg/ml) over night at RT. After it is washed with PBS Laminin (1:1,000) is added and incubated over night at 37°C. From the next day it can be stored up to one month at 4°C.

Cells are expanded in a N2-supplemented DMEM/F-12 medium with B27 (1:1,000), EGF (10ng/ml) and bFGF (10ng/ml) whereas B27, EGF and bFGF are renewed daily. Every third day the cells are fed with 1:3 fresh medium and are passaged every 3-4 days. For passaging 0.025% trypsin is added and incubated at 37°C until the cells are detached. An equal volume of trypsin inhibitor is added and the cells will be collected with basic medium. After centrifuging (300xg, 5min) the calls are replated 1:2-1:3 in fresh complete medium.

For transplantation the cells are proceeded as for the passaging but the cells are resuspended in Cytocon buffer. A 40qm cell strainer is used to exclude clumped cells. Cell are then counted and diluted to a concentration of 100,000-150,000 cells/ql. They're kept on ice during the transplantation session and are replated to asses cell survival

II. Transplantation procedure

In order to avoid a graft rejection the treatment with immunosuppressants need to be started one day in advance with half dose of cyclosporine s.c. (5mg/kg bodyweight).

During the surgical process the animals are anaesthetized by spontaneous inhalation of a mixture of N2O and O2 (70%:30%) with 1-3% halothane. After induction of anesthesia the skull is shaved and treated with antibiotics. Then the skull is fixed into a stereotactic frame with the incision bar at -3.3mm.

The skin is then opened and the skull membranes are removed. After localizing Bregma the desired coordinates can be adjusted (e.g. for injection into rat striatum the coordinates are A-P: 0.0mm, Lateral - 3.0mm and Ventral -5.0mm). A drill is used to make a borehole into the desired position, whereas the borehole is incomplete. The last part of the skull is removed with forceps to not injure the brains surface with the drill. The cells are then filled into a Hamilton syringe and the brains surface is set as Ventral: 0.0mm. The needle is moved down within one minute to the chosen depth and stay there for two minutes before injection of the cells. The cells are injected within one minute and before removing slowly the needle five minutes have to pass to let the cells settle.

After the Hamilton syringe is removed it need to be cleaned immediately to avoid a clumping of the cells, which could disable the needle. The skin is then sutured and treated again with antibiotics. On the day of the transplantation 10mg cyclosporine per kg bodyweight need to be injected subcutaneously. The injection needs to be continued every other day until perfusion.

III. Insights into the cooperation project

The cooperation project was a transplantation study of human iPS cells in young and aged rats following stroke. The rats were subjected to a distal MCAO with 30 minutes bilateral CCA occlusion. 48hours after stroke 300,000 cells per animal (100,000 cells/ql) were transplanted into two injection sites in the ipsilateral cortex to the stroke lesion. Several kinds of behavior tests were done to assess the improvement of disabilities in the different groups. 8 weeks after transplantation rats were sacrificed by transcardiac perfusion with PFA.

The young animals were divided into two groups. The animals in both groups were subjected to stroke, whereas one group is iPS transplanted and the other one received the cell culture medium (vehicle group). The aged animals were divided in three groups. Two groups were subjected to the distal MCAO, whereas again one group received cells and the other group the vehicle. The third group had a sham-stroke operation and vehicle transplantation. All animals received 10mg cyclosporine per kg bodyweight every second day to have the same status of the immune system in all animals.

As behavior tests staircase test, stepping test, rotarod test and cylinder test were used. For the staircase test the animals are placed in Plexiglas boxes with sugar pellets for evaluation of the gripping function in the paws. In the stepping test the animals are slowly guided over 90cm of table to test the motor skills of the right and left front leg. Rotarod tests the animal ability to remain on a stick that rotates with increasing speed. In the cylinder test the animals are videotaped during 5-10 minutes to evaluate disturbance in the motor skills of their paws.

For the staircase and the rotarod test a pre-training of the animals was necessary in order to obtain baselines of their individual skills. For stepping and cylinder use of both paws was assumed to be the baseline.

After perfusion the brains were dehydrated in 20% sucrose and 30µm thick coronal sections were cut on a freezing microtome. The sections were stored in a cryoprotective solution at -20°C until further processing for immunohistochemistry. Staining was performed on free-floating staining in glass vials. The sections were stained for HuNu, NeuN, DCX, beta-III-tubulin, SC121, Ki67, ED1 and Iba-1. With the several stainings it was possible to assess the stroke volume, the survival of the grafted cells, the ratio of the transplanted cells that differentiated to immature or matured neurons, remaining stem cells within the graft and the status of inflammation in the stroke lesion and in the injections site.