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STEMS

PRE-CLINICAL EVALUATION OF STEM CELL THERAPY IN STROKE

Specific Targeted Research or Innovation Project
Thematic Priority 1
Life sciences, genomics and biotechnology for health

Final Report

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Project coordinator: Dr. Brigitte ONTENIENTE
Project coordinator organisation: Inserm

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1. PROJECT EXECUTION

1.1 SUMMARY DESCRIPTION OF PROJECT OBJECTIVES

Given their expected capacity to self-renew and differentiate efficiently into the desired cell type, clonal populations of stem cells (SC) promise to produce beneficial effects in a number of diseases. Several studies already indicate that SC transplantation has a therapeutic potential in stroke, with sources of SC that include embryonic, foetal and adult SC, and lines derived from teratoma (Liu et al., 2000; MacDonald and Howard, 2002; Wei et al., 2005). To date, two cell types of human origin have been used for SC therapy in stroke, bone marrow-derived SC (Bang et al., 2005) and cell lines. The NT2N line (Layton Biosci., Inc.) was derived from a human testicular germ cell tumor. NT2N cells have an exclusive commitment to the neural lineage after retinoic acid treatment, and have been extensively validated in pre-clinical models of stroke without any teratoma or tumour formation reported. A Phase I trial involving 12 patients demonstrated the safety, and partial functional improvements in some patients. A Phase II study showed a global trend towards improvement (Kondziolka et al., 2005). The ReN001 cell line, was developed by ReNeuron from human fetal neuroblasts immortalized with the c-mycERTAM® technology (Stevenato et al., 2009). A Pilot trial with 12 patients is planed in collaboration with the Glasgow University Hospital (UK).

These studies converge to confirm the feasibility and safety of SC therapy in stroke patients. They also demonstrate the need for developing improvements both at cell source and patients recruitment levels. All cell sources come with advantages and disadvantages that should be evaluated. In fact, much crucial information is still lacking before SC transplantation becomes a clinical reality. For instance, the standardisation of the conditions to regulate SC proliferation and differentiation to produce regionspecific grafts need to be better defined; changes in their properties induced by transplantation into lesioned brain structures are poorly understood, as is the full extent of functional improvement at longterm post-stroke delays.

The STEMS project specifically aimed at determining the extent and limits of SC therapy in stroke in order to pave the way for clinical therapeutic trials. This objective had to be achieved through the completion of six successive and complementary tasks:

(1) the definition of the standard experimental conditions for proliferation, guided differentiation, and mass-production of SC-derived products for transplantation,
(2) the identification of the best transplantation protocol with regards to location, number of transplanted cells, and post-stroke delays, in a rat model of focal ischemia,
(3) the control of safety and compatibility aspects,
(4) the quantification of the effects of SC transplantation on functional impairments
(5) the transposition of the optimal experimental conditions defined in rodents to non-human primates,
(6) the definition of the relevant human cell therapy product and operating procedures to be applied to stroke patients.

1.2 CONTRACTORS INVOLVED AND COORDINATOR'S CONTACT DETAILS

INSERM: Institut National de la Santé et de la Recherche Médicale (Coordinator), France
ULUND: Lunds Universitet, Sweden
SDU: University of Southern Denmark, Denmark
KI: Karolinska Insitutet, Sweden, *partner until May 2008*
CEA: Commissariat à l'Energie Atomique, France
Cellartis: Cellartis AB, Sweden
LIN: Leibniz Institute for Neurobiology, Germany
IEM: Institute of Experimental Medicine, Academy of sciences of the Czech Republic, Czech Republic
IT: Inserm Transfert SA, France

Coordinator's contact details
Brigitte Onteniente, I-STEM, INSERM UMR 861/AFM/UEVE, 5 rue Henri Desbruères, 91030 Evry cedex, France
Email: brigitte.onteniente@inserm.fr

1.3 WORK PERFORMED AND RESULTS ACHIEVED

This project aimed at defining the potential of SC therapy in stroke and, therefore, had a strong pre-clinical orientation. Accordingly, the workplan was organized as a longitudinal multidisciplinary study implemented from human stem cell culture to the analysis of graft fate and effects in animal models of stroke with non-invasive approaches similar to those used in humans. The main secondary objectives were to:

- define the most efficient cell source, by comparing human embryonic (hESC) and human adult (hANSC) stem cells,
- define the optimal differentiation stage for transplantation,
- define gender differences,
- develop the proof-of-concept of stem cell therapy for stroke at non-human primate level.

The first 18-months could be divided in three periods with clear objectives. The first 3 months of the workplan have resulted in a major achievement that was not perceived as such initially, the **standardization of procedures among partners**. It was quickly apparent that, although bearing the same name (ES cell culture protocol, transient middle cerebral artery occlusion (tMCAO) in rats, animal strains, behavioural tests), the protocols used in our consortium varied so that it was difficult to compare results and reach constructive conclusions. Standardize of protocols was achieved through exchanges of researcher between groups (3 one-week stays, 1 two-weeks stay). This has been beneficial not only for the STEMS project, but also for the international competitiveness of our groups and for European stem cell research as a whole. Exchanges and regular meetings particularly benefited to our young fellows that could start building long-term relationships for their future carrier in neurosciences.
This 3-months period was also devoted to establish **management guidelines**, develop the website, elaborate the graphic chart and publish leaflets, and to obtain the necessary legal authorizations to perform the work.
During the next 15 months, up to the first activity report (M18), two main objectives were reached, the **development of a robust protocol for differentiation of human ESC-derived neural progenitors**, and the **characterization of a genuine human ANSC line, the NNC1 line** provided by Partner 2.

During this 18-months period, three events occurred that resulted in modifications of the workplan. Firstly, the NNC1 line turned out to be a pericyte-derived, pluripotent cell line, with low capacity to engage into the neural lineage to form neurons. Secondly, in 2006, Takahashi and Yamanaka had described the possibility to derived **pluripotent SC (iPS)** from murine somatic (therefore adult) cells. The iPS field developed exponentially during the next two years and we considered that iPS cells can be compared to ANSC. In May 2008, the Steering Committee decided to include hiPS into the workplan in order to fulfill the comparison with hESC. Thirdly, the primate studies started with an important delay due to moving of CEA to a new site. A 6-months prolongation of the STEMS contract was requested to the EU and obtained. In spite of these adjustments, the overall objectives were not modified.

The main scientific and technical challenges the STEMS consortium had to face during this 42-months period are reported below.

1.3.1. Preparation of transplants

Undifferentiated cells

For comparison purposes, the consortium have started working with 2 different cell sources, hESC from the SA001 line developed by Cellartis and the CCTL14 line available at IEM, and the hANSC derived from human brain biopsies of the subventricular zone (NNC1 line) available at ULUND. Interestingly, the in vitro characterization (ULUND) of the NNC1 line revealed that the NNC1 cells primarily differentiate into mesenchymal phenotypes. At best, their progeny expresses some neural-related proteins without displaying the full phenotype of neurons or glia (manuscript submitted). This was confirmed by transplantation studies performed by LIN in order to identify possible changes in phenotypic orientation induced by grafting into the post-ischemic brain and by SDU into the intact striatum, showing that NCC1 differentiate into adipocyte-like cells following grafting.

HESC culture protocols have been optimized at INSERM and IEM on the basis of pre-existing know-how that lead to reproducible *en routine* production of neural progenitors of the desired differentiation stage for the two hESC lines. A main issue being the standardization of cells differentiation stages in the transplants, this was addressed by KI and ULUND with the identification and analysis of several neuralizing molecules, such

as Mash1, Neurogenin2 and NeuroD1. Viral constructs have shown a certain degree of efficacy on hESC for Mash1 but not on the NNC1 line, confirming a non-neural phenotype.

In the course of this first period, a major advance has been achieved in the field of hANSC by the groups of Shinya Yamanaka (Takahashi et al., 2007) and James Thomson (Yu et al., 2007) with the generation of induced pluripotent stem cells (IPS). IPS are ESC-like stem cells obtained from somatic cells with overexpression of varying cocktails of transcription factors involved in ESC pluripotency and self-renewal. Considering the importance of this discovery with regards to clinical applications, we decided to include IPS in the STEMS project as an alternative source of hANSC. Two hiPS lines were derived from commercially available human lung fibroblasts (MRC5 and IMR90, ATCC) with lentiviral-mediated transduction of Oct4, Sox2, Lin28 and Nanog cDNA according to Yu et al. (2007). The hiPSIMR90 (XX) and hiPSMRC5 (XY) lines were assessed for expression of self-renewal and pluripotency markers, and for formation of embryoid bodies.

Major advances have been performed by Cellartis in up-scaling amplification and banking procedures for hESC and hiPS. This point was crucial with regards to the possibilities to translate our experimental results in clinical practice. The vitrification protocol developed for hES cells was successfully transferred to both hiPS cell lines. The efficacy of the freeze/thaw procedure was validated by analysis of the chemical content of the cells after 10 consecutive passages. Robust results without karyotypic alterations were obtained, confirming the efficacy of the processes. Shipping procedures have been developed for both hESC/hiPS vitrified or frozen vials, and differentiated progenitors and hiPS were live shipped from INSERM to Cellartis using a specific temperature-controlled package. Cellartis also improved banking protocols with the development of a human feeder cell line derived from fibroblasts from umbilical cord, the hUWIL feeders. Compared to mouse MEF feeders, the hUWIL increase the proliferation ratio by 33 times. The hUWIL were successfully tested with both hiPS lines at Cellartis and INSERM. A manuscript will be submitted for publication. Cellartis also developed a new procedure for enzymatic passaging of the cells that allows synchronization and routine production of great amounts of cells.

Differentiation protocols.

TU production from hESC and hANSC was initially secured by INSERM (hESC, SA001 line from Cellartis) and ULUND (NNC1 hANSC line from NeuroNova). INSERM adapted the stromal cells-derived inducing activity (SDIA) protocol established by Perrier et al. (PNAS, 2004) for the generation of dopaminergic neurons in order to obtain GABAergic neurons. GABAergic medium spiny neurons represent the main population of the striatum, the region lesioned by tMCAO in rodents. Replacement therapy after striatal lesion should aim at producing a majority of GABAergic neurons in the transplants. A first study provided the time-course of differentiation of SA001 hESC into neurons with a GABAergic phenotype.

From a genomic study, KI identified the transcription factor Mash1 as a potent neuralisation inducer on ESC. INSERM tried to elaborate a fusion protein with Mash1 coupled to the cell transduction vector TAT-PTD. A number of deletions and mutations in the sequence did not allow the construction of a relevant TAT-Mash1 plasmid. ULUND added a number of molecules with neurogenic activity, such as Ngn2, Tbr and NeuroD in the developing brain.

Transplantation was performed in parallel to observe modifications of the commitment by the post-ischemic environment (see below). As it occurred, this procedure was long (up to 3 months), although it could be shortened by addition of specific cytokines and trophic factors (in particular BDNF) in the culture medium. This is described in Seminatore et al., 2010. The protocol was applied to the monkey ESC line ORMES-18 (Rhesus macaque) in prevision of allografting in *Macaca fascicularis* for the last step of the project.

In a second phase, an improved protocol for differentiation of hESC into neural progenitors was developed at INSERM. This protocol, named the “NSC protocol“, allows large-scale production of highly homogeneous populations of neural precursors. INSERM, LIN and IEM have extensively characterized NSC phenotypes with PCR, FACS, and immunohistochemistry. Importantly for transplantation, NSC pools expressed no markers of undifferentiated or non-neural cells. NSCs can be frozen and thawed on demand for transplantation, or be further differentiated. Established for the hESC lines SA001, the NSC protocol has been successfully transferred to the CCTL14 (IEM) hESC line and to hiPS.

Conclusions

In conclusions, all planned tasks concerning the preparation of transplants were fulfilled. A perivascular progenitor cells from the adult human brain with the characteristics of pericytes and mesenchymal stem cells was identified, showing that the adult human brain harbor progenitor cells with multilineage capacity. A robust protocol for differentiation of two ESC lines (SA001, XY, and CCTL14, XX) and two hiPS lines (hiPSIMR90, XX, and hiPSMRC5, XY) that allow large-scale production of homogeneous pools of NP. NSC-

derived NP can be differentiated *in vitro* into GABAergic neurons. This capacity is kept *in vivo* after transplantation (see WP2). The NSC protocol avoids tenuous characterization of the NP pools looking for proliferation or pluripotency signs. We therefore are confident that the NSC protocol is a robust one to apply to either hESC or hiPS for production of clinical grade pools of transplants.

1.3.2. Fate and integration of transplanted cell

Transplantation studies have started after standardization of protocols for stroke models between INSERM, LIN and IEM (M1-3). The study of the fate and integration of transplanted cells was the largest task of the project. Successful stem cell therapy relies on survival and adequate interactions of grafted cells with the host brain. A number of aspects had to be addressed to understand the efficacy of the approach at structural level and correlate it with functional observations.

Site of transplantation

A major conclusion, reached independently by the 3 transplanting teams, was that, in contrast to reports in the literature, cell transplantation in stroke is deleterious when the cells are transplanted into the peri-lesional tissue. This was observed with ANSC, SA001- and CCTL14-derived neural progenitors and was confirmed with GFP-expressing neural stem cells. Increased lesion size was likely due to disruption of normal tissue by the graft and important glial and immune reactions linked to cell death around the injection site, and was correlated with additional functional deterioration.

The size of the lesion was also identified as a major determinant of cell fate, with significantly reduced graft survival in very large lesions that corresponded to permanent occlusion of the MCA, i.e. which encompassed the striatal and cortical territories of the artery and had extended to parts of pre-optic and hypothalamic areas. An increased formation of teratoma from surviving grafts placed in large lesions was noted (Seminatore et al., 2010) with grafts of early neural progenitors. Noteworthy, grafts placed in the host parenchyma developed less and were less well integrated than grafts placed into the lesion cavity or at the graft-host interface. Reduced development is likely due to physical constraints. Poor migration into the host brain might result from active glial reaction around the graft and production of non-permissive molecules by the host. The described pathotropism of the lesion was also observed, with migration of transplanted cells and increased neuronal differentiation towards the lesion.

Timing of transplantation

This point could not be addressed due to time constraints related to the incorporation of hiPS in the project. However, clinical transfer of hESC or hiPS therapy in stroke patients will, at first, be performed on a limited cohort and very likely with a safety endpoint, i.e. without taking the post-stroke delay as a primary inclusion criterion. As an example, the three main trials published to date, performed with autologous mesenchymal SC (Bang et al., 2009) or with immortalized neural cell lines (Kondziolka et al., 2005), included patients with post-stroke delays ranging from 2 months to 2 years. Such variability is not reproducible in rodents and the Steering Committee decided to focus on hiPS studies to complete the work within the remaining time.

Fate of transplanted cells

The organization of the Consortium and skills and materials available allowed the correlation of histological findings with magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS) and, ultimately, with functional assessments. This was also performed, although to a lesser extent, in monkeys.

Both the lesion and grafts were visible on T2 weighted images using a 3T magnet during the first weeks post-transplantation, and using a 4,5T magnet (graft) up to 4 months post-transplantation, the longer time-point studied. T2 MRI with 3T magnet was the lower limit to accurately quantify the graft volume. Without pre-labeling, the graft core appeared with a density slightly higher than the host grey matter. Cell labeling with iron-oxide nanoparticles before transplantation was useful to observe the cells into the host parenchyma post-mortem. However, nanoparticles produce important artifacts due to intense hyposignal on gradient echo NMR, and the procedure was not retained.

The main objective of this part of the workplan was to define the optimal differentiation stage of neural progenitors (NP) for transplantation. This was inseparable from the functional effects of the procedure. A first series of transplantation with hESC-derived NP allowed a precise description of the behaviour of transplanted cells as a function of their differentiation stage using the SDIA method. Each batch of NP was analyzed before transplantation by quantitative RT-PCR for a set of markers that included pluripotency, proliferation and neuralisation markers. This kinetic study identified four types of transplants, "early-NP", that

still expressed pluripotent markers, "mid-progenitors", with no pluripotency larkers but still proliferative, "late progenitors", with significant expression of neural markers but with important proliferative activity, and neuronal precursors, with strong expression of neural markers and light expression of neuronal markers. As expected, grafts of early progenitors consistently produced teratoma. Grafts of mid-progenitors produced large amounts of neurons arising from rosettes (neural crest-like structures characteristic of the SDIA protocol) similar to the ones observed in cultures. Highly proliferative late progenitors generated tumor-like overgrown grafts that eventually damaged the host brain. Neuronal precursors generated ideal grafts, of limited size that filled up the lesion cavity and produced migrating neuroblasts. Interestingly, neuronal precursor grafts recapitulated a correct neurogenesis program, with a first wave of neurons followed by the production of astrocytes from bi-potent, nestin-positive cells. After 3 months, clusters of DARPP-32 neurons, identifying GABAergic medium spiny neurons, were observed in the grafts, specifically correlated with patches of tyrosine hydroxylase-positive fibers from the host brain. This demonstrated that the dopaminergic afferents of the host brain had recognized, and possibly contacted, the GABAergic neurons of the graft. This work was reported in Seminatore et al. (2010).

A similar investigation was performed with CCTL14 hESC using the embryoid bodies' differentiation technique, with FACS follow-up of the differentiation, which showed the kinetics of pluripotent markers loss and differentiation markers appearance. As expected, immature grafts produced teratoma, and more mature grafts resulted in nice grafts with migrating neuroblasts and neurons.

Considering the interesting nature of undifferentiated NNC1 (pericytes), one additional experiment will be performed to investigate their relationships with host endogenous neural niche and possible involvements in arteriogenesis. Transplanted NNC1-derived NP produced nestin-positive progenitors that frequently co-expressed GFAP.

The exploration of molecular mechanisms that sustain graft integration have been approached unsuccessfully by proteomic analysis of graft and peri-graft tissues. Although a large number of proteins were found differentially expressed, the phylogenetic similitudes between *Rattus norvegicus* and *Homo sapiens* species did not allow identification of the origin of the proteins. MR spectroscopy was successfully used to acquire spectra of metabolites concentrations for several proteins involved in metabolism and signal transduction (N-acetyl-aspartate, choline, creatinine, glutamate, glutamine, etc). Four months after MCAO, MRS revealed that the concentrations of brain metabolites in grafted animals had nearly resumed values found in unlesioned animals. Furthermore, an increase was observed in contralateral hemisphere, in transplanted a well as in control animals, which might be due to plasticity changes related to the ischemic insult in the opposite hemisphere.

During the second reporting period, transplantations were performed using neural progenitors or neuronal precurors differentiated with the NSC protocol. The homogeneity of the cells within transplants was greater, and the protocol was applied to hESC and hiPS lines. The rapid doubling-time of NSC and possibility to freeze/thaw without alterations allowed shipping of batches among the 3 "grafting centers" of the consortium. Comparative studies could be rapidly completed for hiPS-NSC with analysis of their behaviour after transplantation into male or female animals. hiPS-NSC graft produced migrating neuroblasts, neurons and, after 2 months, started producing DARPP-3-positive neurons. They did not contain immature or pluripotent cells and did not produce teratoma or hyperproliferative grafts.

Conclusions

In conclusion, this part of the work displayed two phases related to the evolution of differentiation protocols. The SDIA method requested several weeks of differentiation to obtain safe transplants. The "embrio¨d bodies" protocol was quicker. Both protocols suffered variations in the differentiation stage of the batches of cells. The NSC protocol brought homogenization and the possibility to generate bulks of identical cells for repeated transplantation studies. In agreement, the time for terminal differentiation into GABAergic medium spiny neurons after transplantation was shortened (2 vs 4 months).

A major observation in the study was the fact that both hESC- and hiPS-derived NP differentiate at their own pace. Terminally differentiated neurons were observed after months, which correlates with the delay for striatal formation during embryogenesis. Both hiPS and hESC recapitulated a normal neurogenesis pattern, with formation of nestin bi-potent progenitors, followed by production of neurons, then astrocytes.

1.3.3. Safety and compatibility issues

Safety

Tumour formation is a major obstacle to ESC therapy. As reported for other cell lines, it was observed with early progenitor grafts, indicating that the ischemic lesion do not modify the proliferative capacities of ESC-derived progenitors. However, tumour formation was no longer observed with more differentiated progenitors, and the critical differentiation stage for safe transplantation was established around 80 days of culture for SA001 with the SDIA method, and at 60 days for CCTL14 with the “embryoid bodies” method. This corresponds to complete down-regulation of the expression of the main pluripotent markers (Sox2, Oct4, Nanog, SSEA-4 and TRA-1-60) and strong up-regulation of typical neuroectodermal markers (NCAM, βIIItubulin, NF70, nestin, CD271, and CD29). No tumor or teratoma were observed with the NSC method, which appears more suitable to clinical applications.

A main concern with hiPS generated with viral vectors is potential re-expression of the transgenes during differentiation and, possibly, after transplantation due to the presence of cytokines involved in the expression of pluripotency transcription factors, such as the basic FGF. To examine this issue, we have monitored, in two different groups (INSERM and IEM), the expression of transcription factors used for reprogrammation (Oct4, Sox2, nanog, lin28) and related to pluripotency, at both gene expression and protein levels. A significantly increased level of nanog mRNA was observed in NP from both hiPS lines before grafting, compared to SA001-NP and CCTL14-NP. However, this was not correlated with protein increase. No expression of undifferentiated marker proteins was observed in transplants. This suggests that potential expression of transgenes do not influence the differentiation pattern after transplantation of committed progenitors.

Compatibility

In order to secure uncompromised survival of human neural SC grafts in rat hosts it was decided early in the project to use triple-therapy with CsA, Aza and prednisolone, a procedure close to the one used in the clinic for intracerebral grafting of Parkinson and Huntington patients, starting the day prior to grafting as the standard immunosuppressive treatment.

Graft survival in the triple immunosuppressed ESC and ANSC recipients was comparable 2-4 weeks after transplantation. However, whereas ESC grafts survived well up till 8 weeks, all ANSC grafts were rejected at 10 weeks. This suggests that ANSC grafts elicited a stronger immune response than ESC grafts, or alternatively, that ESCs and ANSCs differed in production of immunomodulatory factors possibly combined with a difference in survival capacity. Despite the difference in long-term survival the immunohistochemical analysis showed that the cellular infiltrates associated with ESC and ANSC grafts in immunosuppressed rat hosts was similar in terms of main infiltrating cell types. Both types of grafts were infiltrated with low numbers of TCR^+ T-cells and activated $CR3^+$/$Iba1^+$microglia/macrophages. We also found evidence of a correlation between immune infiltration and graft differentiation, with fewer immune cells infiltrating the rosettes than surrounding graft areas that contain more differentiated cells. This correlates with quantification of the expression of HLA-ABC markers that showed increasing expression of HLA-ABC with non-neuronal differentation of the NCC1 cells, whereas the more differentiated SA001 cells remained HLA-ABC immunonegative, consistent with reported findings that electrically active neurons express no or negligable levels of MHC class I antigen (Neumann et al. 1995, Science 269:549-52).

To better qualify the innate side of the rejection process, we performed in vitro co-cultures of hiPS-NSC with rat cortex primary cultures. Neurons rapidly developed out of "transplanted" hiPS-NSC. After a few days, microglial cells from the rat culture were observed engulfing human neurons, in the absence of blood-derived cells or cytokines. This work is in progress, and suggests that host resident microglia plays a role in the rejection process. This hypothesis is supported by the observation that triple therapy was insufficient to ensure longterm survival of ANSC grafts, indicating that non-T-cell-mediated mechanisms, possibly involving NK cells and microglia-macrophages, participate in the rejection reaction.

Conclusions

In conclusion, transplantation across a human-to-rat cross species barrier has posed larger than expected problems in terms of immune rejection. The later, however, have been circumvented for periods of up to 8 weeks, by use of triple-therapy with cyclosporin A, azathioprin and methylprednisolone. The transplant rejection, the speed of rejection and the density of the immune infiltrate appear to depend on the following factors:

- the graft source; hESC and hiPS grafts enjoyed a prolonged survival with few graft-infiltrating immune cells, while NNC1 grafts were rejected more rapidly,

- the differentation stage of the grafted ESCs: this influences the strength of the immune infiltration, with the least differentiated part of the graft frequently completely devoid of immune cell infiltration,
- daily use of immunosuppressive therapy.

Non-T-cell dependent effector mechanisms contribute to the rejection.

Although, it is an open question if differences in the levels of HLA-ABC expression would influence ESC rejection across a human-rat species barrier, high levels of HLA-ABC are known to render histocompatible human grafts more susceptible to rejection in human recipients.

1.3.4. Functional outcome

The Consortium has tested a battery of behavioural tests to identify the most relevant to our experimental paradigm. Tests address global (neurological scores, Rotarod, Treadmill) or discrete (limb-placing, tapered beam-walking, staircase) motricity, sensory-motricity (neurological score, adhesive removal test limb-placing) and cognition (neurological scores, staircase, Morris water maze, passive avoidance). Tests were distributed within the Consortium taking into account each Partner's skills and technical possibilities.
Despite the repartition of tests among partners, significance of differences among grafted and non-grafted animals was not achieved due important spontaneous recovery. A return to pre-ischemia levels of skill was observed for all cognitive and sensori-motor tests after a variable amount of time (from 15 days to 3 months) in non-grafted animals. Although the time-course of recovery was quicker in transplanted animals in some of the tests, no significant differences were noted. This probably reflects a reorganisation of the brain circuitry with time. Only animals with very large lesions did not show this spontaneous recovery. The poor survival rate of grafts in such lesions did not allow statistical analysis.
Significant improvements of motor deficits were observed in the apomorphin-induced rotation test. This pharmacological assay does not allow the animal to use adaptive strategies.

Conclusions.

In conclusion, we revealed here an unexpected problem in analysing the long-term effects of grafted cells in the post-ischemic brain. The rat brain adopts dramatically efficient compensatory strategies when lesioned. Despite this bias, we showed for the first time that hiPS-NSC-derived grafts induce long-term improvements of deteriorated motor functions.

1.3.5. Non-human primate studies

Non-human primate studies were planned as the ultimate pre-clinical proof-of-principle of the potential of SC therapy in stroke. As such, they were subjected to sufficient evidence of this potential in rodent studies. Monkey studies started at M18 on the basis of progresses in differentiation protocols and evidence of GABAergic neurons in the grafts. The objectives were to develop a primate model of ACA occlusion and validate the efficacy of transplantation with primate ESC (iPS were not available at that time) using correlated functional, imaging and post-mortem histology.
Several attempts were made to develop a model of ACAO in *Macaca fascicularis* with a non-invasive approach that consisted in injection of microparticles with a catheter inserted into the ACA from the safenous vein. The two first macaques developed small lesions with immediate significant reduction of locomotor activity. However, recovery was rapid and only 30% of the deficits remained after 24 weeks. The two next macaques received larger amounts of microparticles and developed bi-lateral lesions due to anastomosis between the right and left ACA territories. Dramatic morbidity led to sacrifice of the animals within two weeks.
The development of the model was stopped and the transplantation program was pursued with excitotoxic lesion of the striatum. The ESC line ORMES-18, from *Macaca mulatta*, was differentiated into NP with the improved SDIA technique (the NSC protocol was not available). The animal was sacrificed 6 months after transplantation to confirm previous MRI evidence of a surviving graft, in the absence of immunosuppression. The graft contained GABAergic neurons, GFAP astrocytes, and developed no abnormal tissue or unwanted structures.
PET imaging was performed with different tracers to evaluate graft metabolism (^{18}F-FDG), and the neuroinflammatory reaction (^{11}C-PK11195, ^{11}C-DPA713, ^{18}F-DPA714, ^{11}C-SCHxxx). The good integration of the graft into the host brain was reflected by lack of inflammation at 4 and 6 months and by a moderately increased uptake of ^{18}F-DG compared to the normal brain.

Conclusions

On a technical point-of-view, the objectives of this task were only partially reached since the non-invasive primate model of stroke could not be established. However, we have validated a number of procedures, including PET tracers to label neuroinflammation. We also demonstrated that ESC-NP grafts can be followed *in vivo* using PET or MRI techniques, with quantification of the evolution of graft parameters and of host reactions.

On a scientifc point-of-view, two important findings were obtained with potential clinical implication. Firstly, we demonstrated that allografts of phylogenetically close species (Macaca mulata ES cells into Macaca fascicularis) can survive without immunosuppression for at least 6 months in the non-human primate brain. Secondly, we have shown that grafted cells from monkey ESC can produce GABAergic neurons at 6 months when transplanted into the lesioned striatum.

On a scientifc point-of-view, two important findings were obtained. Firstly, we demonstrated that allografts of phylogenetically close species can survive without immunosuppression. Secondly, we have shown that grafted cells from monkey ESC can produce GABAergic neurons when transplanted into the lesioned striatum.

1.3.6. The most efficient protocol for SC transplantation in stroke

This task started with two cell sources, the SA001 hESC line provided by Cellartis and the NNC1 hANSC line available at ULUND. As explained in the report, the NNC1 line turned out to be a pluripotent cell line with preferential commitment to mesenchymal lineages, and comparisons of the effects with hESC would have been misleading. Fortunately, the discovery by Shinya Yamanaka of the possibility to induce pluripotency in adult somatic cells offered an exciting alternative to hESC. The 6-months prolongation of the project allowed completion of the studies with hiPS, and comparisons with previous data obtained with the SA001 and CCTL14 hESC lines.

Globally, the two hESC lines behaved quite similarly with respect to adaptation to differentiation culture. Neural progenitors were initially produced using either the embryoid bodies + POL plating technique or the MS5 + POL plating technique to form neural rosettes. The analysis of neural progenies during differentiation revealed a similar pattern of evolution of neural, then neuronal, markers, for the two hESC lines. With the elaboration of the ‘NSC” protocol, the differentiation became less tedious and time-consuming, and large pools of homogenous NP were produced for subsequent transplantation studies. Again, the two hESC lines complied with the NSC protocol, providing proof of the necessary robustness for clinical translation.

Human iPS have been derived from a number of sources and with a number of protocols. So far, however, little is known about their therapeutic potential in stroke. The two hiPS lines developed for STEMS presented the morphology and all markers required to be a self-renewal, pluripotent source for SC therapy. Both hiPS lines nicely adapted to the NSC protocol, allowing transplantation studies to start immediately after derivation and the characterisation steps. Interestingly, male and female hiPS were available and we could include gender issues in the comparison of their therapeutic effects.

The various results obtained during these 42 months show that hiPS and hESC behave quite similarly. The survival of the hiPS-NP grafts, their size, their differentiation capacities and their functional effects were comparable to SA001 and CCTL14 hESC-NP grafts. We conclude that, considering the ethical concerns raised by hESC in some countries, hiPS represent the optimal source for stem cell therapy in stroke and other neurological disorders.

1.4 ACHIEVEMENTS

The use of SC with multipotent properties has become a challenging research field for most clinical areas. It is of particular importance in disciplines that desperately lack treatment options, such as brain disorders and lesions. In this context, stroke - or ischemic cerebrovascular disease, is an important target. Stroke accounts for roughly half of the patients hospitalised for neurological diseases, and is associated with a large proportion of the health care costs in Europe. Until now, all the neuroprotective approaches that have yielded positive results in animal models of stroke have proven ineffective in clinical trials. Thrombolytic therapy, the only FDA-approved approach to limit the extent of stroke-induced neural degeneration, is effective when given within 3-4 hours after stroke (Hacke et al., 2008). Thrombolytics have associated risks of haemorrhage

and fewer than 5% of patients with ischemic stroke receive the treatment.

The STEMS project aimed at defining the therapeutical potential of SC therapy in stroke by addressing a number of roadblocks that ranged from elaboration of an optimal protocol of differentiation of SC into neuronal precursors to the validation of non-invasive investigation tools in non-human primates. The ultimate goal was to bridge the gap between experimental research and the clinic. The final sum of results show that SC therapy with pluripotent SC could be safely applied to stroke patients and improve the outcome, provided that a number of rules are understood and followed. The work also revealed the limits of animal models in terms of functional assessments and model development in large animals.

1.4.1 Defining the optimal protocol for transplant preparation

Stem cell based regenerative medicine is generally based on adult stem cells and embryonic stem cells. The term adult stem cells also includes foetal cells and stem cells from the umbilical cord blood, since the term embryonic stem cells is restricted to cells derived from the inner cell mass of the blastocyst. The main challenge of the regenerative medicine consists in the dilemma that both forms of stem cells display opposing pros and cons: Embryonic stem cells have the capacity to differentiate into virtually all kinds of somatic cells. However this capacity is attended by the danger of teratoma formation (as again confirmed by results of our consortium) by undifferentiated cells and therefore secure protocols for pre-differentiation prior to transplantation are requested. In addition, embryonic stem cells are derived from supernumerous embryos and that evokes ethical concerns. Further, this implies that hES derived cells always represent an allogeneic graft for potential recipients possibly inducing immunological tissue rejection.

In contrast, adult stem cells can be used as autologous grafts from the body of the patient and thus, not evoking immunological reactions and also harbouring a very limited tumour risk. The main forms of adult stem cells represent bone marrow stem cells (BMSC) and hematopoietic stem cells (HSC). Cryopreserved umbilical cord blood cells (UCB-cells) also contain HSC and several groups have shown that mesenchymal stem cells can also be cultured from UCB.

Both, BMSC and HSC are committed to the mesodermal germ layer and therefore per definition are not capable for the generation of cells derived from the ectoderm like neurons. However, in the last years it became obvious that BMSC represent more complex cell populations as previously recognised. It was demonstrated that bone marrow cells contain sub-populations of stem cells expressing marker proteins typical for embryonic and neural stem cells (Kucia 2006; Sauerzweig 2009). Nevertheless, the differentiation into neuronal cells after transplantation into the adult CNS has not been robustly demonstrated yet. However, many studies underscore the great potential of both HSC and BMSC to confer neuroprotective and remodelling effects, mainly by the secretion of cocktails of growth factors. Since the main advantage of adult stem cells consists in their autologous character when used in regenerative medicine, the full potential of HSC and BMSC is probably best evaluated in clinical studies rather than in basic studies, when human cells are grafted into rats. STEMS has therefore concentrated on the potential of embryonic and induced pluripotent stem cells (human iPS-cells). Human iPS cells are now the most promising candidate since this form of stem cells opens the way for production of autologous specifically pre-differentiated cells. This can be achieved by the induction of fibroblast from the patient. Since iPS-cells in principal behave like ES-cells, many results from the ES-cell research hopefully can be applied directly to the iPS-cell field. Our results confirm this assumption, since the STEMS-consortium demonstrated that iPS cells alike ES-cells differentiate primarily into neurons after delivery into stroke-damaged rats.

Besides the pioneering work performed with foetal tissue, the history of SC therapy with truly pluripotent SC (i.e. forming teratoma after transplantation into the immuno-compromised rodent) can be divided into two phases.

The first phase corresponds to ESC

With the development of ESC research, an impressive number of groups have aimed at understanding the mechanisms of pluripotency. Several molecules and pathways have been identified as factors affecting the molecular mechanisms that govern stem cell self-renewal as well as stem cell fate, either proliferation or differentiation (Kochegarov et al., 2009 ; Tsiftsoglou et al., 2009). In parallel, groups have aimed at refining differentiation protocols to reach the desired phenotypes for specific cell replacement therapy. We have shown that, in contrast to populations of progenitor cells from multipotent neural SC, which often leads to the generation of glial cells (mainly astrocytes), with only a small proportion differentiating into neurons, transplanted ESC-derived progenies tend to generate a majority of neurons. However, multipotent cells with great self-renewal capacity also come with the predictable side-effects, formation of teratoma (Solter et al., 2006) and of overgrowths (Brederleau et al., 2006; Chung et al., 2006; Aubry et al., 2008).

Occurrence of teratoma was reported independently of the origin of the ESC, i.e mouse, non-human primate or human, and with allo- or xenografts (see Seminatore et al., 2010 for references) -although a higher incidence was reported in allograft situations (Erdö et al., 2003). To avoid teratoma, much effort has been devoted to the elaboration of differentiation protocols that allow maximal homogeneity of the transplant (Brederleau et al., 2006) or to cell sorting prior to transplantation to eliminate non-neural progenitors (Chung et al., 2006; Guillaume et al., 2006; Tabar et al., 2005). We have added to these studies evidences of implications of several neurogenic molecules in the differentiation process of neural progenitors (Falk et al., 2007; Roybon et al., 2009; Roybon et al., 2010). Our systematic analysis of the evolution of pluripotency markers during differentiation of hESC prior to transplantation, correlated with *in vivo* observations performed after transplantation, identified the minimal stage of differentiation with the SDIA technique to avoid teratoma formation (Seminatore et al., 2010). It is noteworthy that no teratoma was ever observed after transplantation of NSC-derived NP, or even of NSC without additional differentiation, emphasizing the crucial advantage of this protocol for the production of SC therapy products.

A more recent series of studies have reported a different risk of tumour formation following transplantation of ESC derivatives into the brain (Tabar et al., 2005; Brederleau et al., 2006; Aubry et al., 2008) that corresponds to maintained proliferation of differentiated progenitors. This process, also referred to as “graft overgrowth”, is characterized by the exclusive presence of neural cells –therefore excluding the classification as teratoma- that proliferate to the extent of provoking compression and disruption of host brain structures. Our comparative analysis of pre- and post-transplantation molecular content of the grafts revealed that the neural progenitors display a bi-phasic profile of proliferation, with re-expression of cell cycle molecules just before terminal differentiation into neuronal precursors (Seminatore et al., 2010). Again, the dramatic consequences of overgrowths can easily be avoided by prolonged differentiation of hESC for several weeks.

The second phase corresponded to iPS

The possibility to derive pluripotent stem cells by reprogramming human somatic cells (Takahashi et al., 2007; Yu et al., 2007) has added a unique SC source to the existing panel. IPSC possess the cardinal features of ESC, pluripotency and unlimited self-renewal, which are definitive assets for the standardized, large-scale, production of therapeutic products. In addition to ESC, iPSC have the potential to overcome immune rejection with "patient-tailored" cell lines or to allow HLA-compatible cell banks. However, the resemblance with hESC also comes with similar obstacles for clinical applications, i.e. teratoma/tumour formation. The study performed with hESC to determine the minimal stage of differentiation requested to avoid side effects was not reproduced for hiPS. In the meanwhile, a new protocol was established, the NSC protocol, that allowed us to burn steps with hiPS and directly proceed with safe transplantation. We were able, within 20 months, to derive two cell lines of hiPS, to perform a full characterisation both of the hiPS and of their NP progenies, and to perform a long-term (4 months) characterisation of their fate after transplantation into the post-ischemic brain. No adverse effects or structures were noted.

1.4.2 SC therapy ameliorates the outcome of stroke

The STEMS project aimed at exploring the potential of human pluripotent SC for regenerative medicine in stroke. The effects of SC transplantation were therefore monitored with clinically-relevant non-invasive functional and imaging approaches that were correlated with post-mortem histology.

Several studies have described the potential of ESC-derived neural progenitors to alleviate the symptoms of neurovascular lesions in stroke, with ESC of rodent (Bühnemann et al., 2006), primate (Hayashi et al., 2006) or human (Wei et al., 2005; Daadi et al., 2008; Takahashi et al., 2008; Theus et al., 2008; Hicks et al., 2009; Zhang et al., 2009; Seminatore et al., 2010) origin. Therefore, our studies with hESC primarily focussed on safety aspects, as described above. These studies were initially short-termed (one month) and were progressively extended to longer periods of time with evidences that human NP require several months to fully differentiate into functional neurons *in vivo*. This observation, correlated with sequential apparition of neurons, then astrocytes, in grafts, indicated that grafted human NPs follow the normal human ontogenesis process after grafting. No de-differentiation of transplanted cells was observed. This is an important point, since the post-ischemic brain releases a number of morphogens or trophic factors that can influence transplanted cells commitment. The only influence of the ischemic environment observed was an increased tendency to form teratoma by immature NP, a cell stage totally irrelevant to clinical settings.

The ultimate aim of cell-based stroke therapy is a long-lasting, fully functional, integration of the graft into the circuitries of the recipient brain. The experience of the STEMS consortium confirms the results of previous studies (e.g. Bühnemann 2006) that grafted cells are frequently organised as clumps and surrounded by endogenous astrocytes that might limit migration outside the graft. This demarcation was vaiable form one

animal to another, and obviously had no detrimental effects with regards to neuronal differentiation, and innervation by host afferents. Results shown this July at the 2010 FENS Forum in Amsterdam indicate that limited migration is also caused by the nature of grafted cells. The group of Alvarez-Buylla showed that NP from the fetal lateral ganglionic eminence remain localised in their site of deposition whereas NP from the homotypic medial ganglionic eminence -which will give rise to striatal areas- are able for migration and freely disperse into the cortex of the recipient brain. In our study, the commitment to a striatal fate is performed before grafting and grafted cells did obtain incoming projections from the brain.

The STEMS consortium allowed the first characterization of 3 new PET tracers selective of the TSPO/PBR (translocator protein 18 kDa /peripheral benzodiazepine receptor), and their comparison to the gold standard TSPO radioligand ^{11}C-PK11195, already in use in clinical imaging trials. The TSPO is known to be upregulated in activated microglial cells as well as in reactive astrocytes. The experimental paradigms used within the STEMS consortium allowed to study the 3 new PET tracers kinetics, both under normal and the excitoxically-lesioned non-human primate brain. A longitudinal characterization of these markers under physiological and various time-points following acute excitotoxic brain injury was possible. The full characterization of these radioligands is still ongoing but the data generated during the STEMS period have already demonstrated their great interest as markers of neuroinflammatory responses associated with the excitotoxic lesion such as early microglial activation and delayed astrocytic reaction. Interestingly, two of the PET tracers (^{11}C-DPA713, ^{18}F-DPA714) appeared to label a rather late stage of the lesion process (9 to 14 days post-lesion) associated with Iba1-positive cell proliferation and GFAP-positive cells recruitment into the lesion core whereas the last one (^{11}C-SCHxxx) appeared to label earlier stages of the lesion (72 hrs post-quino) characterized by MRP8-positive cell proliferation within the lesion core and almost no astrocytic reaction. All three radioligands proved to be largely superior to ^{11}C-PK11195 in detecting neuroinflammatory reactions at all stages of the lesion.

Descriptions of successful engraftment of hESC-derived neural progenitors are usually limited to demonstrations of graft-derived cells of neuronal or glial phenotype. Little is known on their integration in the host circuitry. In the perspective of regenerative medicine, this is a crucial objective to achieve. Although no electrophysiological or tract tracing studies were performed in the STEMS project, connections between grafted and host cells were suggested by the topographical correlation of graft-derived DARPP-32 neurons and host-derived tyrosine hydroxylase dopaminergic fibers. This reconstruction of the normal circuitry in the basal ganglia correlates with the functional recovery observed in grafted animals. The relationships between both events now remain to be clarified.

1.4.3 HiPS, the best source for SC therapy in stroke ?

The possibility to derive ESC-like iPS by reprogramming human somatic cells (Takahashi et al., 2007; Yu et al., 2007) have opened unprecedent perspectives in the treatment of neurological disorders with SC therapy. This breakthrough provided a deft approach to the production of patient-specific SC lines and a practical mean of developing haplotyped banks of SC suitable for HLA matching in allograft. As ESC, iPS cells come with a number of concerns concerning their safety and ability to form terminally differentiated neuronal populations. These concerns were primarily addressed in the STEMS project.

Human iPS have been used in models of neurological disorders to replace lost neurons in a model of Parkinson's disease (Wernig et al., 2008) and to derive motoneurons from normal fibroblasts (Karumbayaram et al., 2009) and from fibroblasts of patients suffering of amyotrophic lateral sclerosis (Dimos et al., 2008) and diabetis type 1 (Maher et al., 2009). The reunion of forces from 3 partner institutions allowed us to rapidly go from development of hiPS lines to functional evaluation of the effects of hiPS-NP transplantation into the post-ischemic brain. The STEM consortium was among the first groups to transplant human iPS cells into the stroke-damaged brain, and the first to describe total safety and functional recovery after transplantation of hiPS-NP into the post-ischemic brain. To date, two articles have described the use of undifferentiated iPS in stroke models, to show the expected formation of teratoma (Kawai et al., 2010) and the reversal of functional deficits after transplantation into the subdural space in association with fibrin glue (Chen et al., 2010). In our hands, iPS were the source of choice for SC therapy in stroke lesions.

1.4.4 Primate studies in stroke

Despite the efforts of the scientific community to find new treatments for stroke during the last decade, all therapeutic strategies validated in animal models have failed in humans, with the exception of rtPA-induced thrombolysis. Several reasons for this conundrum have been highlighted during the STAIR (Saver et al., 2009) roundtables, and recommendations for future investigation of the therapeutic potential of drugs or approaches were issued, among which validation in non-human primates. In contrast to rodents, old world

monkeys are phylogenetically close to humans. They have a gyrencephalic brain, the ratio of grey/white matter is identical, the vascular anatomy and the blood flow threshold for ischemic infarction are comparable to humans. In addition, neurological deficits following stroke in non-human primates display high similarities with humans. Tests performed in rodents mainly address motor and sensory-motor functions, leaving aside the disruption of cognitive ability, such as memory dysfunction, topographical disorientation, decreased attention, and diffuse intellectual impairments that characterize stroke patients (Caplan et al., 1985). Non-human primates allow suitable behavioural test batteries and imaging studies with which to accurately assess the pattern of neurological abnormalities caused by cerebral ischemia. Finally, xenografting poorly reflects the clinical situation, and allografting is possible with monkey ESC or iPS (Liu et al., 2008).

Stroke models have been developed in non-human primates with mechanical occlusion by electrocoagulation (Tamura et al., 1981; Cinelli et al., 2001), intraluminal filament insertion (Longa et al., 1989) or ligature (Welsh et al., 1987; Nagai et al., 1999). Models based on endogenous clot formation involve injection of autologous or heterologous pre-formed fibrin/blood clots (Zhang et al., 1997; Kudo et al., 1982; Zhang et al., 2005), microemboli (Atochin et al., 2004), or in situ clot formation with Rose Bengal (Watson et al., 1985). In order to reproduce thrombo-embolic cerebral infarction (75 % of ischemic stroke), we attempted to develop an original, totally non-traumatic model of arterial occlusion, by release of microparticles at brain level through endo-vascular catheterism with a peripheral approach. On a surgical point of view, the procedure was a success, with rapid awakening of the animals and total absence of side effects. However, the lesion size appeared a parameter difficult to standardize, and more animals would have been necessary to fully establish the model.

Although developing a minimally-invasive primate model of stroke proved to be difficult to establish within the time-frame of the STEMS consortium, the entire procedure was completed in 10 minutes in the last 2 animals, vs 30-90 minutes in the first ones. In particular, angiographic imaging of quality improved very significantly the speed of the surgery. The hyperselective catheterization of the anterior cerebral artery was possible in 100% of the cases. The main difficulty was due to variability of blood vessels anatomy in the non-human primate specie selected for this work (Macaca fascicularis), which often consisted of a single trunk for both ACA and required further distal catheterization. Even after the use of an hyperselective unilateral embolization technique, the particular anatomy of the ACA in Macaca fascicularis monkeys (presence of an azygos artery vascularising the two hemispheres), impaired our ability to create a strict unilateral ischemia. This, in turn, resulted in a clear bilateralisation of the oedema (100% of the 5 cases), as demonstrated by the pre-mortem MRI obtained in the last 2 animals and the histological observations conducted in the first 3 cases which always resulted in the severe behavioural deficit observed with other stroke models.

Transplantation studies performed with excitotoxic lesion of the basal ganglia in two macaques were also successful. Allografts survived for 6 months in the absence of immunosuppressive treatment, raising hope that allografts of HLA-compatible NP will survive in Humans. GABAergic neurons were observed in the graft and no astrocytic encapsulation was noted. Imaging allowed the quantification of lesion size, of graft size, and post-mortem co-registration of histological parameters with MRI allowed comparison of imaging and structural data with voxel-size precision. To our knowledge, this is the first time that such techniques are applied to SC transplantation in stroke.

The PET tracers studied in this program will undoubtedly find various applications in the clinic as well as in the industry as a mean to assess and quantify neuroinflammatory responses in vivo. Provided that some additional observations (ongoing work) could be generated demonstrating the specificity of their interaction with a particular cellular target (activated microglia vs. reactive astrocyte), these PET radiotracers will serve as biomarkers of (neuro)inflammatory reactions in preclinical and clinical experimental paradigms. Data generated within the STEMS consortium support the potential clinical use of these tracers not only in stroke, but also other neurodegenerative diseases associated with neuroinflammatory reactions such as Parkinson's and Alzheimer's diseases. The same radioligands may also be used in other non-neurological disorders like AIDS, autoimmune diseases or various cancers.

In conclusion, although the ACAO model could not formerly be established, the use of an excitotoxic striatal lesion model in the non-human primate allowed us to achieve two main goals of STEMS: 1) the development and first validation of new PET radiotracers, specific of the neuroinflammatory responses and 2) assessing the feasibility of using ES cells in the severely lesioned non-human primate brain.

1.5 IMPACT

The STEMS project fully responded to the general objectives of the Life Science and Health Priority of FP6 by

- integrating multidisciplinary research to enable a strong interaction between technology and biology, and improve European scientific and industrial competitiveness,
- defining pre-clinical protocols for rapid translation to the clinic in order to bring major improvement of the quality of life of European citizens,
- taking into account gender and ethic issues, and raising public awareness on these matters by adequate dissemination plans.

A first objective was to facilitate the integration of both public and private research capacities across Europe to increase coherence and achieve critical mass. The STEMS project was ambitious and objectives could not be reached without strong coherence and coordination of tasks among partners. Firstly, efforts were put to homogenize the procedures for stroke modelling and behavioural analysis of functional deficits in rodents. Strains and approaches have been standardised and exchanges of researchers performed to achieve the homogeneity necessary for comparison of data. Similar exchanges were made for SC culture and differentiation protocols. Each group now has access to a sum of tools and knowledge that might widen considerably the individual possibilities of development at technical and conceptual levels. All participants also benefited from the opportunity to expose and train their research personnel to new methods in complementary fields of stem cells and stroke research. The exchange of scientific and technical personnel also contributed to increased mobility among young researchers and better cross-cultural understanding within the European Community.

Secondly, a number of seminars and events were organized to give further chances to young partners to present their data, either orally or on posters. Finally, several articles have been written in common and will open the way to further collaborations.

Importantly, exchanges included common meetings with the two other networks granted with STEMS, the STROKEMAP and STEMSTROKE projects, respectively coordinated by Catherine Verfaillie and Zaal Kokaia. These fruitful exchanges further widened our vision of “stem cells in stroke” research and have created a new community in the field. Altogether, this community is the largest in Europe, and has reached an international level of scientific competitiveness.

A second objective was to improve industrial competitiveness. STEMS had carefully considered the necessity of including SMEs with the highest degree of knowledge and technology in the stem cells field. Few SMEs fulfilled these requirements at that time. The choice of Cellartis AB was based on the excellence of their products, already used by a number of European laboratories, and by the will of the company to develop the necessary technology for a rapid translation of SC-derived products into clinical products. Although not directly involved in research activities, the inclusion of Inserm Transfert should also be regarded as directly linked to our concern to improve industrial development. Inserm Transfert has specialised in technology transfer and project management, and its activity has already led to the creation of 8 new biotechnological companies. Both Cellartis and Inserm Transfert have brought consistent plus-value to the consortium in terms of possibilities of industrial innovation, licensing, and translation of the new knowledge into effective therapy and clinical practice.

For instance, Cellartis developed several technologies, among which the SCED™461 has been patented. The vitrification procedures and the hUWIL feeders have been adopted by INSERM and daily afford efficacy and reproductibility to our culture process. Cellartis has put great efforts in developing protocols that can be translated into GMP practices and accelerate the translation of SC therapy for stroke to the clinic. All processes have been already translated to hiPS, a highly competitive field for patents.

A third objective was to increase European health level. The European Community plays a particular role in promoting health protection and disease prevention. STEMS complied with this obligation by addressing one of the most devastating diseases of the modern world. To the number of patients that directly suffer from stroke each year, one should add the distress and anxiety of relatives and the social burden created by the disability and loss of autonomy of these patients who require constant care and support. STEMS was created to provide clinicians with a number of data concerning the benefits of several cell sources in neurovascular lesions. We have reached the conclusion that both human ESC and iPS can be a relevant source for the derivation of clinical grade transplants. We have established the conditions of teratoma/tumour

formation by derivatives of pluripotent stem cells and have shown that safe grafts can be easily produced by monitoring the proliferation profile of NP during their differentiation process. We have also shown that the commonly used anti-cancer agent temozolomide (Temodar™ or Temodal™) can stop proliferation and transplant overgrowth *in vivo*, if needed.

Three important developments for the translation of the approach to clinical settings include the NSC protocol, the validation of a series of procedures for large-scale amplification of pluripotent SC, banking and shipping, and the validation of a number of non-invasive techniques to investigate the graft fate and its relationships with the host brain.

Altogether, we can now propose a readily usable, robust, protocol for the production of pre-clinical grade neural progenitors for transplantation. All protocols are amenable to GMP procedures.

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2. DISSEMINATION AND USE

2.1 EXPLOITABLE KNOWLEDGE AND ITS USE

Scale-up of human embryonic stem cell production (Cellartis)

Stem cell bulk production and shipping procedures were considerably improved by Cellartis, and new clinical-grade feeders are being implemented. In particular, Cellartis has further standardized and optimized the previously developed single cell enzymatic dissociation (SCED) culture protocol. Results obtained during the first period of STEMS provided confirmed that the passage interval and the passage ratio are clearly dependent on hES cell line and its specific population doubling time. Importantly, Cellartis optimized the freezing protocols used for the product in the framework of the STEMS project. Detailed Exploitation plans of the SCED technology and the product based thereof, SCED™461, have been provided in separate exploitation plans.

2.2 DISSEMINATION OF KNOWLEDGE

1. Publications/Manuscripts

Partner(s) involved	Authors/Year/Title/Journal	Under Preparation/submitted/published
KI	*Falk et al. (2007)* High-throughput identification of genes promoting neuron formation and lineage choice in mouse embryonic stem cells. **Stem Cells** 25:1539-1545;	Published
ULUND	*Roybon L, Mastracci TL, Ribeiro D, Sussel L, Brundin P, Li JY.* (2010) GABAergic differentiation induced by Mash1 is compromised by the bHLH proteins Neurogenin2, NeuroD1, and NeuroD2. Cereb Cortex. 20:1234-44. *Roybon L, Deierborg T, Brundin P, Li JY*. (2009) Involvement of Ngn2, Tbr and NeuroD proteins during postnatal olfactory bulb neurogenesis. Eur J Neurosci. 29:232-43. *Roybon L, Hjalt T, Stott S, Guillemot F, Li JY, Brundin P.* (2009) Neurogenin2 directs granule neuroblast production and amplification while NeuroD1 specifies neuronal fate during hippocampal neurogenesis. PLoS One. 4:e4779.	Published
IEM	*Kozubenko N., Turnovcova K., Kapcalova M., Butenko O., Anderova M., Rusnakova V., Kubista M., Hampl A., Jendelova P., Sykova E.* (20010) Analysis of in vitro and in vivo characteristics of human embryonic stem cell-derived neural precursors. Cell Transplant. 19(4):471-86.	Published
INSERM	*Aubry L, Bugi A, Lefort N, Rousseau F, Peschanski M, Perrier AL.* Striatal progenitors derived from human ES cells mature into DARPP32 neurons in vitro and in quinolinic acid-lesioned rats. Proc Natl Acad Sci U S A. 2008 Oct 28;105(43):16707-12.	Published
INSERM, SDU, IEM	*Seminatore C, Polentes J, Ellman D, Kozubenko N, Itier V, Tine S, Tritschler L, Brenot M, Guidou E, Blondeau J, Lhuillier M, Bugi A, Aubry L, Jendelova P, Sykova E, Perrier AL, Finsen B, Onteniente B.* (2010) The postischemic environment differentially impacts teratoma or tumor formation after transplantation of human embryonic stem cell-derived neural progenitors. Stroke 41(1):153-9.	Published

ULUND	*Paul G, Ozen I, Brundin P et al.* The human adult brain harbours progenitor cells with mesenchymal and neuroectodermal potential.	Submitted
INSERM	*Nissan X, Aubry L, Boissart C, Perrier A, Peschanski M, Benchoua A.* MiR 125 acts in conjunction with BMP and activin/nodal inhibitors to promote early neural specification of human embryonic stem cells.	Submitted
SDU, INSERM and all	*Finsen B, Ellman D, Onteniente B, and the STEMS Consortium.* Factors determining the survival of intracerebral human stem cell grafts. REVIEW	In preparation
SDU, INSERM	*Ellmann D, Lambertsen K, Seminatore C, Mirza B, Polentes J, Onteniente B, Finsen B.* Correlation between Immune infiltration and differentiation stage of human embryonic stem cell-derived grafts in immunosuppressed rat hosts.	In preparation
SDU, ULUND	*Ellmann D, Mirza B, Lambertsen K, Roybon L, Paul G, Brudin P and Finsen B.* Prevention of rejection of adult human neural NNC1 cells following intracerebral transplantation to the striatum of adult rats.	In preparation
INSERM, IEM, LIN	*Polentes J, Jendelova P, Cailleret M, Braun H, Tropel P, Seminatore C, Kozubenko N, Brenot M, Baldauf K, Côme J, Tournois J, Reymann K, Sykova E, Stephane V, Onteniente B.* Human induced pluripotent stem cell-derived neural progenitors alleviate stroke-induced functional impairments.	In preparation
CEA	Use of ^{11}C-SSRXXX as a marker of microglia in neuroinflammatory processes associated with excitotoxic striatal lesions in primates.	In preparation
CEA	Use of ^{11}C-DAP713 and ^{18}F-DPA714 as a marker of microglia in neuroinflammatory processes associated with excitotoxic striatal lesions in primates.	In preparation
CEA	Use of 3D-reconstruction toolboxes in non-human primate models of ischemic damage.	In preparation

2. Meeting Presentations

Partner(s) Involved	Conference	Oral Presentation/ Poster	Title
INSERM, CEA	8° Coll. Soc. des Neurosciences, Montpellier, ***France***	Poster	*Seminatore C, Polentes J, Itier V, Aubry L, Bugi A, Delzescaux T, Dhenain M, Hantraye P, Perrier A, Onteniente B (2007).* Correlations between functional and structural observations after transplantation of human embryonic stem cell-derived progenitors into the ischemic rat brain.
INSERM, CEA	Brain '07, Osaka, ***Japan***	Poster	*Seminatore C, Polentes J, Itier V, Aubry L, Bugi A, Delzescaux T, Ghenain M, Hantraye P, Perrier A, Onteniente B (2007).* Survival and functional effects of human embryonic stem cell-derived progenitors transplanted into the ischemic rat brain
INSERM (Co-	FISH-ESC: First International Symposium on Human Embryonic	Oral	The STEMS project: Pre-clinical analysis of stem cell therapy in

organiser of the Meeting)	Stem Cell Research, 31.01-02.02.2008, Evry, ***France***	Presentation	stroke
INSERM, SDU, IEM, STEMS consortium	FISH-ESC: First International Symposium on Human Embryonic Stem Cell Research, 31.01-02.02.2008, Evry, ***France***	Poster	*Polentes J, Tine S, Seminatore C, Ellman D, Jendelova P, Lambertsen KL, Kozubenko N, Blondeau J, Sykova E, Finsen B, Onteniente B and the STEMS consortium.* Efficient immunosuppressive treatment promotes graft survival and allows tumour development from xenografted hESC in models of stroke.
INSERM	FISH-ESC: First International Symposium on Human Embryonic Stem Cell Research, 31.01-02.02.2008, Evry, ***France***	Poster	*Seminatore C, Polentes J, Tine S, Bugi A, Blondeau J, Perrier A, Onteniente B (2008).* Fate and functional effects of hESC-derived progenitors transplanted into a model of cerebrovascular lesion.
LIN	Symposium on Neuroprotection and Neurorepair, Cerebral Ischemia and Stroke, 17-20 May 2008, Magdeburg ***Germany***	Poster	Stem cell therapy in stroke
IEM	Symposium on Neuroprotection and Neurorepair, Cerebral Ischemia and Stroke, 17-20 May 2008, Magdeburg ***Germany***	Poster	Fluorescence-activated cell sorting for the transplantation of human embryonic stem cell-derived neural precursors after MCAO in rats. 5th International Symposium on Neuroprotection and NeurorepairCerebral Ischemia and Stroke. Magdeburg, May 17-21, 2008
INSERM, SDU, IEM, STEMS consortium	Symposium on Neuroprotection and Neurorepair, Cerebral Ischemia and Stroke, 17-20 May 2008, Magdeburg ***Germany***	Poster	*Polentes J, Tine S, Seminatore C, Ellman D, Jendelova P, Lambertsen KL, Kozubenko N, Blondeau J, Sykova E, Finsen B, Onteniente B and the STEMS consortium.* Efficient immunosuppressive treatment promotes graft survival and allows tumour development from xenografted hESC in models of stroke.
INSERM	6th FENS Forum of European Neuroscience, Geneva ***Switzerland***, 12 – 16. July 2008	Chair, organiser of Technical Workshop	Stem cell therapy in stroke
SDU, INSERM, ULUND	6th FENS Forum of European Neuroscience, Geneva ***Switzerland***, 12 – 16. July 2008	Oral Presentation	*Finsen B, Ellmann D, Mirza B, Lambersten K, Polentes J, Roybon L, Brundin O, Onteniente B (2008)* Suppression of immune rejection of human neural stem cells grafted into striatum of intact and stroke lesioned rats
LIN	6th FENS Forum of European Neuroscience, Geneva ***Switzerland***,	Oral Presentation	Evaluation of functional recovery

	12 – 16. July 2008		
IEM	6th FENS Forum of European Neuroscience, Geneva ***Switzerland***, 12 – 16. July 2008	Oral Presentation	In vivo stem cell tracking in brain and spinal cord
Cellartis	ISSCR 2008, Philadelphia, ***USA***	Poster	Supportive culture protocols for the scale up of human embryonic stem cell production
Inserm	The STEMSTROKE Meeting, Cardiff, ***UK***	Oral Presentation	*The STEMS network*
Inserm	The STROKEMAP Meeting, Leuwen, ***Belgium***	Oral Presentation	*The STEMS network*
LIN, Inserm	The STROKEMAP Meeting, Leuwen, ***Belgium***	Poster	Neuronal differentiation of human iPS cells in a cortical primary culture
IEM	ISSCR 7th Annual Meeting, Barcelona, ***Spain***, July 8-11,2009	Poster	*Kozubenko N., Turnovcova K., Kapcalova M., Sindelka R., Kubista M., Hampl A., Jendelova P., Sykova E.* Analysis of in vitro and in vivo characteristics of human embryonic stem cell-derived neural precursors
IEM	Strokemap meeting Leuven, ***Belgium***, September 17-18, 2009.	Poster	*Kozubenko N., Turnovcova K., Kapcalova M., Sindelka R., Kubista M., Hampl A., Jendelova P., Sykova E.* Human embryonic stem cell-derived precursors: their in vitro and in vivo characterization
IEM	Joint Conference of the Czech and Slovak Neuroscience Societies, Prague, ***Czech Republic***, November 1-4, 2009.	Poster	*Turnovcová, K., Kozubenko, N., Kapcalová, M., Jendelová, P., Syková, E.* Fluorescence-activated cell sorting for the transplantation of human embryonic stem cell-derived neural precursors after MCAO in rats
IEM	Analytical Cytometry V, Olomouc ***Czech Republic***, September 5-8, 2009.	Poster	*Turnovcová K, Kozubenko N, Kapcalová M, Jirák D, Burian M, Jendelová P and Syková E.* Fluorescence-activated cell sorting for the transplantation of human embryonic stem cell-derived neural precursors after MCAO in rats
Inserm	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010, ***Lille***, France	Oral Presentation	iPS for stroke
SDU	Neuroimmune Interactions, Courses and workshops organized by the Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), University of Minho, Braga, Portugal. COST Action	Poster	*Ellman D., Mirza B., Lambertsen K.L., Roybon L., Paul G., Brundin P., Finsen B. and the STEMS Consortium.* Graft survival in immunosuppressed rats following intracerebral transplantation of

	Neurinfnet. June 22-27, 2009.		human stem cells.
SDU	Joint PhD course and 25 years jubilee-meeting for the Danish Society for Neuroscience, Copenhagen, Denmark. November 26-27, 2009.	Poster	*Ellman D., Lambertsen K. L., Mirza B., Roybon L., Paul G., Brundin P., Finsen B. and the STEMS Consortium*. Intracerebral survival of human NNC1 cells transplanted into immunosuppressed rats
Inserm	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010, ***Lille***, France	Poster	*Polentes J, Seminatore C, Boissard C, Feyeux M, Onteniente B, Itier V.* Fine tuning of hESC-derived neural stem cells characterization by coupled structural and functional approaches
IEM	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010 in Lille, France	Oral Presentation	Cell therapy: a comparison of adult and embryonic stem cells
LIN	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010, ***Lille***, France	Oral Presentation	MSC and ESC before and after transplantation into stroke damaged rats
SDU	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010 in Lille, France	Oral Presentation	Safety & compatibility issues
Cellartis	SC-stroke – European symposium on Stem Cell Research for Stroke, 4 – 6 May 2010 in Lille, France	Oral Presentation	Advancements in human embryonic stem cell technologies toward clinical applications
LIN	FENS Meeting, Amsterdam, The Netherlands	Poster	Braun H, Cailleret M, Onteniente B, Reymann K. Neuronal differentiation of human iPS-cells in a cortical primary culture
Cellartis	ISSCR 6th Annual Meeting, Philadelphia, US, June 10-13, 2008	Poster	Johannisson J, Ellerström C, Nordb M, Rydström, I, Kilmare E, Hyllner J Strehl R: Successful scale-up of hu embryonic stem cells using human feeders isolated from the umbilical cord
IEM	40th Annual meeting of SFN San Diego, October 2010	Abstract submitted	*Kozubenko N, Turnovcova K, Seminatore C, Jirak D, Onteniente B, Jendelova P, Sykova E* Using human induced pluripotent stem cell-derived neural precursors in a rat model of stroke
IEM	ISCT –Europe 2010, Belgirate, Italy, September 2010	Abstract submitted	*K.Turnovcova, N. Kozubenko, B. Onteniente, D Jirak, Eva Sykova, P. Jendelova* The induced pluripotent cells-derived neural precursors in the focal brain ischaemia treatment in rats.

3. Any other dissemination activities

Partner(s) Involved	Please describe
IT/Inserm /all	Website www.stemsproject.eu
Inserm	Press release, 14. March 2007 concerning the launch of the project STEMS
Inserm	15. May 2007, Les transversales santé, Oral Presentation, Paris Development, Cellules souches et maladies neurodégénératives
IT/ Inserm	Contribution (description of the consortium and its objectives) to the European Commission's Catalogue comprising all FP6 projects relevant to New Therapies (2007)
Inserm	Organisation of the WP3 Workshop on Stem cell therapy in Stroke. FENS Symposium, Geneva, 2008.
IT/ Inserm	Contribution (description of the consortium and its objectives) to the European Commission's Catalogue comprising all FP6 projects relevant to Rare Diseases
ULUND	Participation to the "Cell Technologies for Medicine" course organised by V.A. Almazov Federal Center for Heart, Blood & Endocrinology, St Petersburg, Russia, within "Russian/Swedish Research Week", May 11-15, 2009
Inserm	Organisation of the public European symposium on Stem Cell Research for Stroke SC-stroke (62 participants) with the participation of SDU, LIN, IEM and Cellartis, 4-6 May 2010, Lille, France
SDU	Ellman D., Mirza B., Lambertsen K.L., Roybon L., Paul G., Brundin P., Finsen B. and the STEMS Consortium (2009). Graft survival in immunosuppressed rats following intracerebral transplantation of human stem cells. Neuroimmune Interactions, Courses and workshops organized by the Life and Health Sciences Research Institute (ICVS), School of Health Sciences (ECS), University of Minho, Braga, Portugal (June 22-27 2009), within the COST Action Neurinfnet. Poster presentation.
SDU	Joint PhD course and 25 years jubilee-meeting for the Danish Society for Neuroscience, Copenhagen, Denmark. November 26-27, 2009.