Proposal/Contract no.: 037526

StemStroke

Towards a stem cell therapy for stroke

Specific Targeted Research Project

PRIORITY FP6-2005-LIFESCIENCEHEALTH-6
Life Sciences, Genomics and Biotechnology for Health

FINAL ACTIVITY REPORT

Period covered: from 1.01.2007 to 31.12.2009

Date of preparation: 15.02.2010

Start date of project: 1.01.2007 **Duration**: 36 months

Project coordinator: Zaal Kokaia
Project coordinator organization name: Lund University (ULUND)

Stroke is a major cause of long-term disability in humans, but effective treatments are lacking. The StemStroke consisted of well-established and highly qualified research teams and it was working with human neural stem cell (NSC) from the foetal and adult brain, and from embryonic stem cells. Cellular and molecular mechanisms regulating the proliferation, migration, survival, and differentiation of the NSC lines after transplantation into the stroke-damaged rodent brain are widely studied. In parallel, StemStroke tried to unravel mechanisms regulating self-repair after stroke through formation of new neurons from the adult brain's own NSCs. The StemStroke explored the morphological and functional integration of grafted and endogenously generated NSCs and their progeny in the stroke-damaged brain, and developed new *in vivo* imaging and behavioural tests, relevant for the human situation, for assessment of stem cell function and recovery of sensory, motor and cognitive deficits. StemStroke aimed to optimize transplantation- and endogenous neurogenesis-based strategies and create an important preclinical protocol which can be rapidly translated into human trials.

Objectives:

The scientific objectives of the project was **to develop novel strategies to repair the brain and restore function after stroke** based on transplantation of NSCs or their derivatives, or stimulation of the production of new neurons from the adult brain's own NSCs. The **specific objectives** were:

- To isolate and characterize NSC lines derived from human ES cells and foetal and adult brain tissue in culture, and to investigate their ability to differentiate into the specific neuron types lost following stroke
- To develop strategies to stimulate neurogenesis from endogenous NSCs in adult brain following stroke
- To evaluate the potential of recently described adherent NSCs for functionally relevant neuronal differentiation in comparison with conventional neurosphere cultures
- To develop non-invasive MR imaging for monitoring *in vivo* survival, migration, and differentiation of endogenous and grafted NSCs after stroke
- To demonstrate whether new neurons, generated from endogenous or grafted NSCs, are morphologically and functionally integrated in the stroke-damaged brain
- To develop a standardized sensorimotor and cognitive test battery for assessment of functional efficacy of grafted and endogenous NSCs after stroke
- To optimize the magnitude of functional recovery induced by endogenous and grafted NSCs after stroke
- To develop the first preclinical protocol for application of stem cell therapy in stroke patients

Partners:

The StemStroke consortium comprised 5 academic research teams and one SME which, together with excellent clinicians in the stroke field, were performing innovative research leading to the first preclinical protocol for application of stem cell therapy in stroke patients. The Principal Investigators representing the corresponding teams were as follows:

Prof. Zaal Kokaia - *co-ordinator* **ULUND** Lund Strategic Research Centre for Stem Cell Biology and Cell Therapy, University Hospital, Laboratory of Neural Stem Cell Biology BMC B10, SE-221 84 Lund Sweden, E-mail: Zaal.Kokaia@med.lu.se

Prof. Austin Smith - **UCAM-STEM** University of CambridgeWellcome Trust Centre for Stem Cell Research, Cambridge, UK.

Prof. Mathias Hoehn - **MPI** Max-Planck-Institute for Neurological Research, In-vivo-NMR Laboratory. Cologne, Germany.

Dr. Liliana Minichiello - **EMBL** European Molecular Biology Laboratory, Mouse Biology Unit, Monterotondo, Rome, Italy.

Prof. Stephen Dunnett - **CU** Cardiff University, Brain Repair Group, School of Biosciences, Cardiff, UK.

Dr.Lilian Wikström - **NEURONOVA** NeuroNova AB, Stockholm, Sweden.

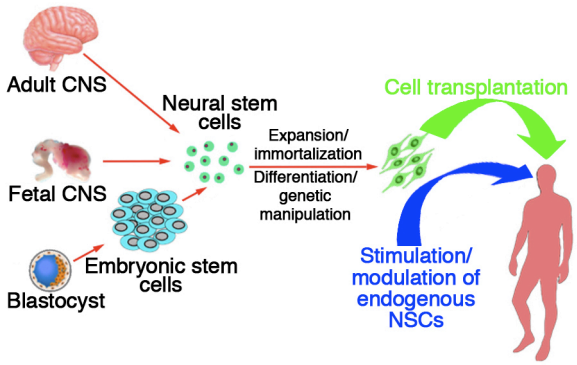
The teams contained top-level expertise in animal models of stroke and MRI-based *in vivo* imaging, stem cell, molecular, and cellular biology, molecular genetics, animal behaviour and psychology, translational research, and clinical cell therapy. The complementarity of expertise within the consortium, together with the intellectual and technological resources available from each partner, ensures efficient and high-quality performance and feasibility of achieving its ambitious S&T goals.

Novelty of the approach:

There were several aspects of the research in StemStroke which were unique and novel: First, the project for the first time attempted to generate NSC lines from human ES cells and foetal brain, and directly, and in the same animal model, compare their capacity to repair and restore function in stroke-damaged brain. Second, the project for the first time explored the potential of adherent NSCs generated from ES cell lines which were produced by Partner 2 and had been shown to be stably neurogenic and non-tumorigenic (Conti et al., 2005). Third, the project for the first time attempted to develop MR imaging and a behavioral test battery for efficient monitoring of stem cell survival, migration, differentiation, and function after stroke, allowing for direct translation to humans. Fourth, the project for the first time developed a preclinical protocol selection of patients for stem cell therapy in stroke based on both findings in animal models and the pathology and symptomatology in patients.

Methodology and techniques:

Two main strategies to generate neurons for replacement in stroke-damaged brain were used in this project (Fig.1):



In the first strategy, NSCs isolated from human embryonic stem (ES) cells or from human foetal brain tissue, were expanded in culture, genetically modified (when needed), and subsequently transplanted into the recipient subjected to stroke. In the second strategy, neurogenesis from endogenous NSCs were stimulated using tools developed in the project, leading to increased survival, migration or maturation of newly formed neurons. The morphological and functional integration of grafted and endogenously generated NSCs and their progeny in the stroke-damaged brain was assessed using immunocytochemistry, anatomical tracing techniques, and patch-clamp electrophysiology. New in vivo MR-based imaging and behavioural test battery, developed within the proposed project, were used to assess stem cell function and recovery of sensory, motor and cognitive deficits. Finally, the transplantation- and endogenous neurogenesis-based strategies were optimized in animal models of stroke and, in close interaction between basic scientists and clinicians, for development of preclinical protocol for stem cell application in stroke.

The work performed and results achieved:

Generation and characterisation of cell line resources for transplantation in stroke:

One of the main topic of the StemStroke is to isolate and generate new NSC lines of human origin and test them in animal model of stroke. During the first half of the project several such lines have been isolated, expanded and characterized *in vitro*. Most importantly, these cells were freely available for the consortium members. As defined with M.1.1. and M.1.2. Partner 2 has expanded, cryopreserved, and provided to Partner 1 a panel of human ES cell- and foetal brain-derived NSCs and trained and transfered technology on human NSC cultures. These cells will be further used in stroke experiments. In addition, Partner 2 has finished characterisation of differentiation of human foetal brain-derived NSCs as planned by M1.4. and as defined by D.1.2. We generated human-derived NSC lines expressing GFP reporter suitable for monitoring NSC differentiation in cultures and after transplantation into the brain. We have published a paper (*Sun et al., 2008,* ***MCN;*** *Sun et al*., ***PLOS One***, 2009) where report on generation and expansion for long-term multiple human foetal NSC lines in monolayer culture without genetic immortalization. This line can be stably transfected to provide reporter lines and readily imaged in live monolayer cultures, creating the potential for high content genetic and chemical screens. However, derivation of similar long-term NS cells from human embryonic stem (hES) cell sources has proven problematic. Furthermore, NS cells appear to have limited capacity to generate diverse neuronal sub-types in vitro and recent experiments in the Cattaneo laboratory suggest that apparent neuronal differentiation after transplantation may arise primarily via cell fusion. This hypothesis was tested by Partner 1 (Cusulin and Kokaia, 2010, ***manuscript***). It was shown that ES-derived mouse NS cells (mNS) fuse with pyramidal neurons after the transplantation in the cortex of rat and mouse newborns. This process is ultimately mediated by microglia, in a two-step manner. We demonstrated that mNS rapidly fuse with microglia, both in vivo and in vitro, and these hybrids in turn fuse with the mature neurons. The phenomenon described here is, to our knowledge, the first report of cell fusion between NSCs and host elements. Overall, we have demonstrated, for the first time, that mouse NS cells fuse in vivo with mature neurons. These findings not only shed a new light on the functions and roles of microglia, but also provide a cautionary note for all researchers doing transplantation studies. The possibility that grafted cells fuse with host cells is something that we hope investigators will address in future studies. In addition, Partner 2 has now derived a different type of adherent NSC lines from both hES cells and human induced pluripotent stem (iPS) cells. These cells are distinct from the foetal NS cells and show features of more primitive neuroepithelial cells. They were therefore named neuroepithelial-like stem (NES) cells. NES cells grow homogenously and can be massively expanded in culture without losing their ability to self-renew and differentiate. NES cells derived from different human ES or iPS cells show consistent features. They are capable of differentiation into neurons, astrocytes and oligodendrocytes *in vitro*. NES cell lines have the potential to provide a renewable source of neurons for basic and applied research, disease modelling pharmaceutical screening, and possibly cell replacement therapy for stroke.

Partner 1 explored how proneuronal transcription factor Pax6 affect the neuronal properties of NSCs derived from fetal human cortex and striatum. We published a paper (Kallur et al., 2008, ***MCN***) where we showed that the Pax6 increased *in vitro* generation of neurons from striatal but not cortical NSCs, derived from 6-9 weeks old human fetuses, without affecting survival and proliferation. Overexpression of mouse Pax6 produced increased numbers of GABA+ and DARPP-32+ (characteristic of striatum) but not glutamate+ neurons (characteristic of cortex). Our data suggest that Pax6 function is conserved between species since its overexpression activates similar genes in mouse and human NSCs. Also, that Pax6 overexpression in striatal NSCs increases the number of neurons but their region-specificity is maintained.

Generation of neurons from endogenous adult brain stem cells in response to stroke

Together with generation of new human-derived NSC lines for transplantation in stroke model, in StemStroke consortium we were exploring the mechanisms of stroke-induced neurogenesis. In accordance with M2.1, we have completed analysis of stroke-induced response in NSCs, endothelial cells and other cellular elements in the SVZ. In recently published manuscript (***Thored et al., 2007, Stroke***), adult rats were subjected to MCAO and hypoxia was studied by injecting Hypoxyprobe-1 during MCAO or few weeks later. We showed that the ischemic insult caused transient hypoxia and early, low-grade angiogenesis, but no damage or increase of vascular density in the SVZ. Our data indicate that the vasculature plays an important role for long-term striatal neurogenesis after stroke. During several months, neuroblasts migrate close to blood vessels through an area exhibiting early vascular remodeling and persistently increased vessel density.

In another study which was not originally planned within the StemStroke project and will be soon submitted (Ahlenius et al., 2010, to be submitted to ***Cell Stem Cell***), we studied the adaptor protein Lnk, which had been described in hematopoiesis whereas its function in the nervous system is unknown. We studied the role of Lnk for NSCs under normal conditions and after insults. We found that Lnk-deficient (Lnk-/-) mice exhibit increased SVZ NSC proliferation after stroke but not under basal conditions or after epilepsy. Lnk inhibited growth factor induced NSC proliferation by reducing phosphorylation of Erk and Akt pathways. Expression of Lnk and growth factors is upregulated in SVZ after stroke but not epilepsy. Our study, for the first time, demonstrated Lnk in adult brain NSCs and presents evidence of its insult-specific involvement as a negative regulator of NSC proliferation.

For further exploration of the effect of anti-inflammatory treatment on endogenous neurogenesis after stroke (M2.7.) and investigation of stroke-induced cellular and molecular changes in SVZ (D.2.2), Partner 1 perform study to determine the origins and quantify the numbers and the proportion of various morphological subtypes of microglia in SVZ at different time points after stroke and to explore whether T-cell-triggered conversion of microglia to MHC-II-expressing, antigen-presenting cells could play a significant role also for stroke-induced neurogenesis in the SVZ (Thored et al., 2009, ***Glia***). We demonstrated increased numbers of activated microglia in ipsilateral SVZ concomitant with neuroblast migration into the striatum following stroke in rats. In the periinfarct striatum, numbers of activated microglia peaked already at 2 weeks and declined thereafter. Microglia in SVZ were resident or originated from bone marrow, with maximum proliferation during the first 2 weeks post-insult. In SVZ, microglia exhibited ramified or intermediate morphology, signifying a down-regulated inflammatory profile, whereas amoeboid or round phagocytic microglia were frequent in the peri-infarct striatum. Numbers of microglia expressing markers of antigen-presenting cells increased in SVZ but very few lymphocytes were detected. The long-term accumulation of microglia with proneurogenic phenotype in the SVZ implies a supportive role of these cells for the continuous neurogenesis after stroke.

In addition to existing Milestones and Deliverables, we have introduced several new ones which were not included in Technical Annex which are in line with the other tasks and overall of the whole project. Among those project, there is one which has been completed (M.2.11) and published (Danilov et al., 2008, ***Glia***). We have investigated ultrastructure (electronmicroscope) of neurofgenic SVZ in adult rodents. We found astrocytes with single cilia and neuroblasts. We also observed mitotic cells, ependymal cells, displaced ependymal cells, and mature astrocytes. In contrast, transient amplifying precursor cells were not detected. The NSCs and neuroblasts had EGF receptor (EGFR) and PDGF receptor alpha (PDGFR) expressed on the ciliary apparatus and were the only cell types incorporating the proliferation marker BrdU. Throughout mitosis, EGFR and PDGFR were associated with the microtubule of the mitotic spindle. Our findings indicate that the NSCs in adult rat SVZ give rise directly to neuroblasts. Our data indicate that in adult rat SVZ during mitosis, NSCs disassemble primary cilium, symmetrically distribute EGFR and PDGFR in the progeny, and directly give rise to neuroblasts.

Another very high profile study, which was not included in original Technical was collaborative work performed by Partner 1 together with Prof. Jonas Frisen's group from Karoliska (Carlen et al., 2009, ***Nat. Neurosci***). We characterized the properties of new cells generated from ependymal cell layer in response to stroke. In this study we showed that ependymal cell quiescence was actively maintained by canonical Notch signalling. Inhibition of this pathway in uninjured animals allowed ependymal cells to enter the cell cycle and produce olfactory bulb neurons, whereas forced Notch signaling was sufficient to block the ependymal cell response to stroke. Ependymal cells were depleted by stroke and failed to self-renew sufficiently to maintain their own population. Thus, although ependymal cells act as primary cells in the neural lineage to produce neurons and glial cells after stroke, they do not fulfill defining criteria for stem cells under these conditions and instead serve as a reservoir that is recruited by injury.

During the second half of the project, we put major emphasis to investigate the role of BDNF-TrkB signalling at different phases of stroke-induced striatal neurogenesis. Partner 4 have established for this purpose an inducible system that allows for the inactivation of trkB in NSCs and progenitors in the SVZ. In particular, a mouse strain (the trkB floxed mouse), which has been previously generated by Partner 4, has been crossed to a mouse transgenic strain carrying a tamoxifen inducible Cre recombinase under the control of a nestin enhancer element (Nestin-Cre-ERT2). Our preliminary data indicate that TrkB-BDNF signalling is involved proliferation as well neuronal differentiation of SVZ progenitors in response to stroke. In addition, to better understand the mechanism of action of TrkB signalling, Partner 4 studied the potency of this receptor tyrosine kinase to modulate synaptic plasticity and activate signal transduction pathways mainly through two phosphorylation sites (Musumeci et al., 2009, ***J. Neurosci***). To identify the molecular pathways required for learning and synaptic plasticity downstream of TrkB, we used highly defined genetic mouse models carrying single point mutations at one of these two sites (Y515F or Y816F) to examine the physiological relevance of pathways activated through these sites for pavlovian fear conditioning (FC), as well as for synaptic plasticity as measured by field recordings obtained from neurons of different amygdala nuclei. We show that a Y816F point mutation impairs acquisition of FC, synaptic plasticity, and CaMKII signaling at synapses. In contrast, a Y515F point mutation affects consolidation but not

acquisition of FC to tone, and also alters AKT signaling. Thus, TrkB receptors modulate specific phases of learning and synaptic plasticity through two main phosphorylation docking sites.

Inflammation and its role for neurogenesis has been also explored and Partner 1 completed analysis of the role of TNFα signalling for endogeneous neurogenesis after stroke (M2.4). The data have been published in accordance with D.2.3. (***Iosef et al., 2008, J. Cereb. Blood Flow Metabol***). In this study we showed that the increased proliferation 1 week after stroke occurs concomitantly with elevated microglia numbers and TNF-alpha and TNF receptor-1 (TNF-R1) gene expression in the SVZ of wild-type mice. TNF receptor-1 was expressed on sorted SVZ progenitor cells from nestin-green fluorescent protein reporter mice. In animals lacking TNF-R1, stroke-induced SVZ cell proliferation and neuroblast formation were enhanced. In contrast, deletion of TNF-R1 did not alter basal or status epilepticus-stimulated cell proliferation in SVZ. Addition of TNF-alpha reduced the size and numbers of SVZ neurospheres through a TNF-R1-dependent mechanism without affecting cell survival. Our results provide the first evidence that TNF-R1 is a negative regulator of stroke-induced SVZ progenitor proliferation. Blockade of TNF-R1 signaling might be a novel strategy to promote the proliferative response in SVZ after stroke.

Morphological and functional integration of neurons generated from grafted and endogenous neural stem cells

In StemStroke consortium we attempted to analyse the functional integration of grafted and newly generated neurons in the diseased brain. We published paper (Darsalia et al., 2008, ***Eur. J. Neurosci***) where we have transplanted NSCs from human fetal striatum and cortex into the stroke-damaged striatum of adult rats. The two types of NSCs exhibited a similar robust survival (30%) at 1 month after transplantation, and migrated throughout the damaged striatum. Striatal NSCs migrated farther and occupied a larger volume of striatum. Human cells outside the transplantation core differentiated, exhibited mature neuronal morphology and expressed mature neuronal markers. Based on these data, human fetal striatum- and cortex-derived NSCs could be considered potentially safe and viable for transplantation, with strong neurogenic potential, for further exploration in animal models of stroke.

Since stroke is age-related disease and major of stroke patients are over 70 year old Partner 1 initiated new study which aimed to explore the properties of NSCs in adult and aged brain (Ahlenius et al., 2009, ***J. Neurosci***). We have compared the properties of NSCs isolated from embryonic lateral ganglionic eminence and adult and aged SVZ in mice using in vivo and in vitro systems, analyzed their gene expression profile, and studied their electrophysiological characteristics before and after differentiation into neurons. We showed a loss of NSCs in SVZ from aged mice accompanied by reduced expression of genes for NSC markers, developmentally important transcription factors, and neurogenic factors. However, when isolated in vitro, the NSCs from SVZ of aged animals have capacity for proliferation and multilineage differentiation, including production of functional neurons, similar to that of NSCs in adult mice, albeit with lower efficacy. These properties are of major importance when considering therapeutic applications of neuronal replacement from endogenous NSCs in the injured, aged brain.

During the second half of the project, inflammation and its role for neurogenesis and functional integration of newborn neurons has been also explored by consortium. For assessment how the environment influences the properties of newborn cells and alters their morphological and elecrophysoiological properties (Jakubs et al., 2008, ***J. Neurosci***), we explored, using confocal microscopy and whole-cell patch-clamp recordings, whether a chronic inflammatory environment affects the morphological and electrophysiological properties of new dentate gyrus granule cells. The brain inflammation was induced by lipopolysaccharide, which gave rise to long-lasting microglia activation. We demonstrated for the first time that inflammation influences the functional integration of adult-born hippocampal neurons. Our data indicate a high degree of synaptic plasticity of the new neurons in the inflammatory environment, which enables them to respond to the increase in excitatory input with a compensatory upregulation of activity and efficacy at their afferent inhibitory synapses.

Development of in vivo imaging and behavioural tests:

The development of new methods for in vivo monitoring of grafted NSCs and newborn neurons as well as assessment of post-stroke behavioral impairments was on e of the important topics in the activity of StemStroke. Partner 3 has characterized temporal profile of macrophage activity in the lesion periphery by MRI, as a function of absence/presence of implanted NSCs. Imaging sequences were established that are sensitive to the susceptibility effects of iron oxides. Different types of ultra small particles of iron oxides were administered intravenously at different time points post stroke and continuous imaging was performed to detect iron containing blood borne macrophages in the brain that arrived from the periphery. Contrary to previous results, no iron induced signal changes were observed in the brain at any timepoint with any employed agent. Final data for this task is currently being collected. Preliminary results with one of the contrast agents tested are currently in press (Farr and Hoehn, 2008, ***Future Neurology***).

In series of experiments, NSCs derived from the human fetal striatum (derived by Partner 1), were expanded by Partner 3as neurosphere cultures, were labelled with superparamagnetic iron oxide (SPIO) particles. We found that the human NSCs readily incorporated the SPIOs without addition of lipofectant agents and that the concentration of contrast agent needed was relatively low compared to previously published reports. The labelled NSCs kept the label after two weeks of differentiation in vitro, thus, immediate dilution of label upon cell differentiation is not to be expected even though dilution will take place due to cell proliferation, cell death and probable passive release of label. Furthermore, scanning at 11.7T of gelatine phantoms loaded with labelled cells, revealed that we could detect as low as 100 cells by MRI. Importantly, the SPIOs did not affect the proliferation, survival and differentiation capacity of the human NSCs in vitro.

Partner 3 has also performed characterization of temporal profile of macrophage activity in the lesion periphery by MRI, as a function of absence/presence of implanted NSCs (M.2.3). Imaging sequences were established that are

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Two large cohorts of rats were trained and tested on separate batteries of motor and sensory tasks (Partner 5). Cognition was not studied at this stage due to the unilateral nature of the MCAO model not being suitable for the study of striatal based cognition. The rats undertook pretraining on specific tasks followed by 30 min MCAO. 24 hours following surgery the rats were MRI scanned in order to confirm lesioning. At the 24 hour time point and at 7 days the rats were also tested on a basic neurological score (Chen et al., 2001), the cylinder test (Schallert, 2006) and the stepping test (Olsson et al., 1995). At 1 months and 2 months following MCAO the rats were assessed using on their respective test batteries. Animals were then perfused and histological analysis of brain sections undertaken, including detailed cell counting using stereological methods. Due to surgery not always producing lesions, an extra group of animals was lesioned at a later date and tested. Stereological analysis of the brains of these animals is underway and all stereological data will be correlated with the behavioural measures outlined in table 1. Correlations will include examining which behavioural measure correlate more closely to cortical degeneration and those which correlate to striatal degeneration. White matter damage will also be examined in relation to its effect on behaviour. In conclusion, from examination of a wide number of motor and sensory tests, it was decided that the tests to take forward and use in a standardized battery are: 1.Paw reaching – staircase; 2.Paw placing; Disengage; 3.Ladder; 4.Apomorphine induced rotations. As these are the most sensitive to deficits following 30 minutes of MCA occlusion, and include both motor and sensori-motor tasks.

Optimisation of neural stem cell transplantation- and neurogenesis-based strategies

Consortium also performed substantial amount of work for optimization of NSC transplantation - and neurogenesis-based strategies. In one of the studies (Darsalia et al., 2010, submitted to ***JCBFM***), we investigated how various steps of neurogenesis are affected by intrastriatal transplantation of human NSCs at different time points after stroke and with different numbers of cells in each implant. Rats were subjected to middle cerebral artery occlusion and then received intrastriatal transplants of NSCs. Transplantation shortly after stroke (48h) resulted in better cell survival than did transplantation 6 weeks after stroke, but the delayed transplantation did not influence the magnitude of migration, neuronal differentiation and cell proliferation in the grafts. Transplanting greater numbers of grafted NSCs did not result in a greater number of surviving cells or increased neuronal differentiation. A substantial number of activated microglia were observed at 48h after the insult in the injured striatum, but reached maximum levels 1 to 6 weeks after stroke. Our findings show that the best survival of grafted human NSCs in stroke-damaged brain requires optimum numbers of cells to be transplanted in the early post-stroke phase, before the inflammatory response is established. These findings therefore have direct clinical implications.

Very importantly, during the second half of the project we developed the protocol for clinical study (***The Lund Stroke Recovery Study*** (LSRS) which aims for understanding and exploring feasibility between clinical and experimental stroke research. The main objective of the present study is to investigate and collect relevant clinical data regarding ischemic stroke patients that may be eligible for neural stem cell therapy. We initiated a two-part study. Part1) a "screening study", in order learn what is the age- and sex-specific annual incidence rate of subcortical and combined subcortical and cortical infarcts. Moreover, to explore how much neurogenic areas with endogenous NSCs are involved in ischemic stroke and what is the severity of stroke in patients with isolated and combined subcortical infarcts. In the second part of the study ("comprehensive study") in a selected set of patients from the abovementioned "screening study", we aim to understand what morphometric changes can be detected on MRI of patients with subcortical infarcts after 3-12 months and what functions are most commonly impaired in patients with subcortical infarcts. We will also assess what changes in clinical impairments in patients with subcortical infarcts can be detected with comprehensive clinical examinations after 3-12 months which of them are worthwhile to improve for enhancement of patients quality of life? Most importantly we are aiming to understand whether patients with subcortical infarcts with/without neurogenic area involvement differ in recovery after stroke. The ethical permit for this study has been obtained (attached as Appendix 3)and we have already enrolled more than 50 patients and the results of this study will serve as bases for Phase 1 clinical trail involving stem cell therapy approaches.

Through the collaboration and subcontract with ***Swedish Institute of Health Economy***, the consortium produced the unique health economy model for stem cell therapy in stroke patients. In this modelling study we evaluated the value of stem cell therapy (SCT) versus standard care among patients with ischemic stroke. The model is driven by functional status as measured by the modified Rankin Scale (mRS). Subtracting the model-generated cost per QALY from the threshold value gives an estimation of the justifiable price of stem cell therapy. This model is currently adjusted to Swedish healthcare system but could be easily changed by taking into account the specificities and costs for any European healthcare system. This model (attached as Appendix 1) will be written as scientific paper and published in

close future. It has already received high appraisal from the president of World Stroke Organization and other stroke experts.

Consortium members have presented their data at many scientific conferences and meeting as lectures as well as oral and poster presentations. The perspectives and road-map of stem cell therapy for stroke patients was discussed in details by the consortium members and invited world-experts in the field of experimental and clinical stroke during the round-table discussion organized by the consortium. The highlights of the discussion and perspectives of the field development will be summarized in ***Int. J. Stroke*** (preliminary agreement achieved with editor-in-chief).

In addition to the original works, consortium members produced several review articles in top scientific journals and financial support of EU through funding of StemStroke consortium was acknowledged (Koch et al., 2009; ***Lancet Neurol***; Lindvall and Kokaia, 2010, ***J. Clin. Invest***; Minichiello, 2009, ***Nat. Rev. Neursci***; Popa-Wagner et al., 2009, ***Ageing Res. Rev***.). In these publications, the knowledge generated from the work in StemStroke consortium has been discussed in relationship with future development of stem cell biology and stem cell therapy for neurodegenerative diseases.

Consortium management:

The consortium did its best to ensure that the it functioed properly and that all deliverables were delivered and milestones were achieved as planned. Moreover, the consortium management made sure that all partners and team members received all necessary information and support, and that annual scientific and financial reports were produced in time and with high quality.

The consortium management structure was as follows: The Scientific Coordinator headed the consortium and was in charge of management. Together with the other Principal Investigators (Partners) and Chief Clinical Advisor, Scientific Coordinator has formed the Steering Group.

Consortium from the very first days of its existence paid a lot of attention to proper management of its work. The project manager has been recruited. In order to improve her efficiency and educate on the issues related to FP7, Katarina Turesson attended FP7-financial & project management course arranged by Europa Media on 27-28 March 2008, in Budapest, Hungary. As defined in M6.2. we set up video system Marratech with main goal is to arrange Steering Group's monthly internet video conferences as well as bilateral scientific discussions. Partner was able to connect to this conference room through consortium homepage (http://www.stemstroke.eu/meeting-place) and participate in the video conference/discussion. Several video conferences have been arranged with useful discussions and decision relevant for the project (D6.2.).

We have also arranged the Clinical Advisory Board (M6.3.) for the discussion on the issues related to translation of basic research data into clinically justified work. The Board has been formed and it is consist of Prof. Olle Lindvall, Prof. Bo Norrving and Prof. Arne Lindgren. Board is headed by Prof. O. Lindvall who has headed the clinical neurotransplantation program in Lund and coordinated a network of research groups. Profs. Norrving and Lindgren are clinicians specialized with stroke and have a strong clinical as well as research background. Prof. Norrving is President of World Stroke Organization and European Editor of "Stroke" and Prof. Lindgren is heading the Lund Stroke Registry which contains clinical and genetic data of more than 3000 patients with stroke. This Board has meat for several time and designed the clinical epidemiological studies which will serve for consortium's ultimate goal of creating pre-clinical protocol for stem cell therapy in stroke patients.

The consortium also established contact (M6.4) with SAFE - Stroke Alliance for Europe, the organization which unites various European patient organisations dealing with stroke prevention and care (www.safestroke.org). We have placed link to their homepage at our homepage and asked them to disseminate information about our consortium.

Consortium arranged total 4 meetings. The consortium's first "kick-off" meeting was arranged in Lund, Sweden on January 15, 2007. All Partners and associates participated in the meeting and we discussed the future plans and ways how to arrange our work and interaction between partners. The consortium's second and third meetings were also arranged in Lund, Sweden (on March 15, 2008 and January 25, 2009, respectively). We have discussed the ongoing work, future plans and preparation for the mid-term and final reports. All Partners and associates participated in the meeting and we have discussed issues related to running project as well as our future plans, modifications to old and new milestones and deliverables, and preparation for mid-term report. During the final meeting which was held in Cardiff, UK (November 26, 2009) a separate session was devoted to a discussion around the preclinical protocol of stem therapy for stroke patients which is based on the data obtained by StemStroke. We have invited a wide range of clinical and rehabilitation specialists, physicians and nurses, and scientists with whom we shared our knowledge which led to this protocol, and defined future directions for possible implementation of our protocol in clinical trials. The pre-clinical protocol developed by StemStroke will presented by the members of the consortium at European and other international scientific and clinical meetings with the clear aim to explore possibilities to translate this protocol into clinical trial for stroke patients. The materials of round table discussion will be summarized as Review paper and published in International Journal of Stroke (preliminary agreement achieved with the editor, Prof. Donnan, who himself participated in the work of round table).

Shortly after the "kick off" meeting Consortium arranged its own webpage (D6.3) http://www.stemstroke.eu. The page contains information about the consortium, describes the participating partners, gives the callender of the events, list of the papers published from the consortium and supported by StemStroke, the information about consortium published or broadcasted in different media sources, lists of available positions within the consortium. The homepage also allows to enter meeting place for carrying out video conferences and contains the folder where password-protected access to the internal documents is available.

Annex - Dissemination of knowledge

The dissemination of results between the PIs is implemented through internet conferences, and with other members through 4 meetings of the consortium which took place in Lund and Cardiff. In addition, e-mail and phone communication is carried out between the coordinator and the other PIs on a regular basis, as required. The consortium has established a website (http://www.stemstroke.eu). The site contains partner contact information, a PDF version of the project, WP description, agendas and instructions for upcoming consortium meetings, and downloadable scientific and financial reports. This part of the website is password- protected for access by consortium members.

In parallel, we have created a publicly accessible part of the web-site to provide information about the StemStroke objectives and goals, to address general issues related to the use of different types of stem cells in medical (and specifically stroke) research and to make advances and developments achieved in the work programme available to the general audience. We have established contact with the EC-supported Stroke Alliance for Europe (SAFE), which organises at the European level all national patient organizations for stroke.

The major form to disseminate results to the scientific community is publications in peer-reviewed scientific journals. We have so far to published data generated by consortium in high-impact journals easily accessible for a wide range of scientists and physicians. Notably, consortium members published several review articles (with acknowledging EU support) which is addressing wider audience and not only specialists in stem cells and stroke. During reporting period, he following articles have been published or are in press with acknowledgement of StemStroke support:

Original and review articles

1. Ekdahl, CT, Kokaia, Z, and Lindvall, O (2009). Brain inflammation and adult neurogenesis: The dual role of microglia. **Neuroscience**, 158:1021-9
2. Hoehn, M, Himmelreich, U, Kruttwig, K, and Wiedermann, D (2008). Molecular and cellular MR imaging: Potentials and challenges for neurological applications., **J Magn Reson Imaging**. 2008.27(5):941-54 (*Review*)
3. Justicia, C, Ramos-Cabrer, P, and Hoehn, M (2008). MRI detection of secondary damage after stroke: chronic iron accumulation in the thalamus of the rat brain. **Stroke**, 39(5):1541-7.
4. Kallur, T, Gisler, R, Lindvall, O, and Kokaia, Z (2008). Pax6 promotes neurogenesis in human neural stem cells. **Mol Cell Neurosci**, 38(4):616-28.
5. Soria, G, Wiedermann, D, Justicia, C, Ramos-Cabrer, P, and Hoehn, M (2008). Reproducible imaging of rat corticothalamic pathway by longitudinal manganese-enhanced MRI (L-MEMRI). **Neuroimage**. 41:668-74.
6. Sun, Y, Pollard, S, Conti, L, Toselli, M, Biella, G, Parkin, G, Willatt, L, Falk, A, Cattaneo, E, and Smith, A (2008). Long-term tripotent differentiation capacity of human neural stem (NS) cells in adherent culture. **Mol Cell Neurosci**.
7. Weber, R, Ramos-Cabrer, P, Justicia, C, Wiedermann, D, Strecker, C, Sprenger, C, and Hoehn, M (2008). Early prediction of functional recovery after experimental stroke: functional magnetic resonance imaging, electrophysiology, and behavioral testing in rats. **J Neurosci**, 28(5):1022-9.
8. Darsalia, V, Kallur, T, and Kokaia, Z (2007). Survival, migration and neuronal differentiation of human fetal striatal and cortical neural stem cells grafted in stroke-damaged rat striatum. **Eur J Neurosci**, 26(3):605-14.
9. Hoehn, M, Wiedermann, D, Justicia, C, Ramos-Cabrer, P, Kruttwig, K, Farr, T, and Himmelreich, U (2007). Cell tracking using magnetic resonance imaging. **J Physiol**, 584(Pt 1):25-30.
10. Thored, P, Wood, J, Arvidsson, A, Cammenga, J, Kokaia, Z, and Lindvall, O (2007). Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. **Stroke**, 38(11):3032-9.
11. Danilov A, Gomes Leal W, Ahlenius H, Kokaia Z, Carlemalm E. and Lindvall O. Ultrastructural and antigenic properties of neural stem cells and their progeny in adult rat subventricular zone. **Glia**, 2009;57(2):136-52.
12. Farr TD and Hoehn M (2008). Perspectives of in vivo magnetic resonance imaging of cell dynamics in the brain. **Future Neurology**. 3: 423-432.
13. Jakubs K, Bonde S, Iosif RE, Ekdahl CT, Kokaia Z, Kokaia M. and Lindvall O. Inflammation regulates functional integration of neurons born in adult brain. **Journal of Neuroscience**, 2008, 28, 12477-88.
14. Carlén M, Meletis K, Göritz C, Darsalia V, Evergren E, Tanigaki K, Amendola M, Barnabé-Heider F, Yeung M.S.Y, Naldini, L, Honjo, T. Kokaia, Z, Shupliakov O, Cassidy R.M, Lindvall, O and Frisén, J. Forebrain ependymal cells are Notch-dependent and generate neuroblasts and astrocytes after stroke. **Nature Neuroscience**. 2009, 12, 259-267.
15. Ahlenius H, Visan V. Kokaia M, Lindvall O and Kokaia Z. Neural stem and progenitor cells retain their potential for proliferation and differentiation into functional neurons despite lower number in aged brain. **Journal of Neuroscience**, 2009, 29, 4408-4419.
16. Koch P, Kokaia Z, Lindvall O, Brüstle O. Emerging concepts in neural stem cell research: autologous repair and cell-based disease modelling. **Lancet Neurology,** 2009, 8, 819-829.
17. Lindvall O, Kokaia Z. Stem cells in human neurodegenerative disorders--time for clinical translation? **Journal of Clinical Investigations,** 2010, 120, 29-40.

18. Popa-Wagner A, Buga AM, Kokaia Z. Perturbed cellular response to brain injury during aging. **Ageing Research Reviews**. 2009. Epub Nov 10.
19. Musumeci, G., Sciarretta, C., Rodríguez-Moreno, A., Al Banchaabouchi, M., Negrete-Díaz, V., Costanzi, M., Berno, V., Egorov, A.V., von Bohlen und Halbach, O., Cestari, V., Delgado-García, J.M., Minichiello, L. TrkB modulates fear learning and amygdalar synaptic plasticity by specific docking sites. **J. Neurosci** 2009, 29:10131-43.
20. Sun Y, Kong W, Falk A, Hu J, Pollard S, Smith A. CD133 (prominin) negative human neural stem cells are clonogenic and tripotent. **PLoS One**. 2009 Epub May 11.
21. Minichiello, L. TrkB signaling pathways in LTP and learning. **Nature Rev Neurosci** 2009, 10(12):850-60.
22. Darsalia V, Allison S.J, Carlo Cusulin C, Monni E, Kuzdas D, Kallur T, Lindvall O and Kokaia Z. Cell number and timing of trans¬plantation determine survival of human neural stem cell grafts in stroke-damaged rat brain (under revision in *JCBFM*).
23. Wood J, Jackson J.S, Jakubs K, Ekdahl C.T, Zaal Kokaia Z, Kokaia M and Lindvall O. Functional integration of new hippocampal neurons born in adult epileptic brain is determined by characteristics of pathological environment (under revision in J. Neuroscience).
24. Farr TD, Seehafer JU, Nelles M, Hoehn M. Challenges towards MR-imaging of the peripheral inflammatory response in the sub-acute and chronic stages of transient focal ischemia. (*submitted*).
25. Ahlenius H, Darsalia V, Iosif R.E, , Torper O, Wood J, Jacobsen S-E, Lindvall O. and Kokaia Z. Adaptor protein Lnk is a negative regulator of brain neural stem cell proliferation after stroke (*manuscript*)
26. Cusulin C. and Kokaia Z. Mouse ES-derived neural stem (mNS) cells fuse with microglia and pyramidal neurons in the neonatal cortex (*manuscript*)
27. Cusulin C, Monni E, Cavallaro M. and Kokaia Z. Generation and characterization of NS cell line from human fetal striatum (*manuscript*).
28. Falk A*, Koch P*, Takashima Y, Brustle O, Smith A. Consistent generation of long-term expandable neuroepithelial-like stem cells from human embryonic stem cells and induced pluripotent stem cells (*manuscript*).

Conferences and meetings:

1. Imaging macrophage infiltration of ischemic tissue is not possible following transient middle cerebral artery occlusion. Abstract and oral presentation. International Society for Magnetic Resonance in Medicine, 16th Scientific Meeting and Exhibition. Metro Toronto Convention Centre, Toronto, Ontario, Canada, May 3-9, 2008.
2. A re-examination of the capability of magnetic resonance imaging to observe macrophage infiltration into ischemic tissue. Abstract and poster. 5th International Symposium on Neuroprotection and Neurorepair: Cerebral Ischemia and Stroke. Herrenkrug Parkhotel, Magdeburg, Germany, May 17-20, 2008.
3. A critical re-examination of the capability of MRI to detect macrophage infiltration into ischemic tissue. Invited presentation. World Molecular Imaging Conference. Acropolis Convention Centre, Nice, France, September 10-13, 2008.
4. Stem cells and cerebral ischemia (stroke): Animal models and clinical perspectives. Meeting of Danish Society for Neurorehabilitation and Danish Stem Cell Research Center, Odense, Denmark, February 22, 2007.
5. Brain plasticity in recovery from stroke: a cellular point of view. 17th ENS meeting- 3rd Conference on Advances in Molecular Mechanisms of Neurological Disorders, Salamanca, Spain, May 19-22, 2007.
6. Stem cell therapy as a therapeutic strategy for stroke. 4th World Congress of the International Society of Physical and Rehabilitation Medicine, Seoul, Korea, June 10-14, 2007.
7. Stem cell-based therapy for ischemic stroke. II International Symposium in Advanced Therapies and Stem Cells, September 21-23, 2007, Rio De Janeiro, Brazil.
8. Neural stem cell therapy for functional recovery after stroke. Silver Congress of International Psychogeriatric Association - Active Aging: Wisdom for Body, Mind and Spirit, symposium "Regenerative Medicine in CNS Disorders", October 14 - 18, 2007, Osaka, Japan.
9. Cell replacement therapy for stroke - how to make it work? EuroNeuro 2008 meeting, to be held January 17-19, 2008, Maastricht, The Netherlands.
10. Stem cells and cerebral ischemia (stroke): Animal models and clinical perspectives. Neuroregeneration neuroplasticity and neurorehabilitation: Joint meeting of Danish Society for neurorehabilitation and Danish Stem Cell Research Centre & Danish Stem Cell Research Doctoral School, February 21, Odense, Denmark.
11. Neural stem cells and ischemic stroke. 5th International Symposium on Neuroprotection and Neurorepair -Cerebral Ischemia and Stroke, May 17-20, 2008, Magdeburg, Germany.
12. Stem cell therapy for stroke from endogenous and exogenous sources. The XXXI Nordic Congress in Clinical Chemistry (NFKK Labmed2008), June 14-18, 2008, Helsinki, Finland.

13. Kallur T, Farr TD, Wiedermann D, Couillard-Després S, Aigner L, Hoehn M. Imaging neurogenesis after stroke in the mouse brain. Abstract and poster. 7th Annual Meeting of the International Society for Stem Cell Research. Centre Convencions Internacional. Barcelona, Spain. July 8-11, 2009.
14. Farr TD, Kallur T, Wiedermann D, Couillard-Després S, Aigner L, Hoehn M. Live imaging of stroke induced neurogenesis in the mouse brain. Abstract and poster. 24th International Symposium on Cerebral Blood Flow, Metabolism, and Function & 9th International Conference on Quantification of Brain Function with PET. McCormick Place. Chicago, Illinois, USA. June 29- July 3, 2009.
15. Farr TD, Kallur T, Wiedermann D, Couillard-Després S, Aigner L, Hoehn M. Abstract and oral presentation. 3D optical imaging for endogenous cell migration after stroke. European Network of Cell Imaging and Tracking Expertise, Educational Workshop on molecular imaging: How does it work and what is new? Congress Center IKEM, Prague, Czech Republic, May 7-8, 2009.
16. Farr TD, Kallur T, Wiedermann D, Couillard-Després S, Aigner L, Hoehn M. Combined in vivo MR and optical imaging of stroke induced neurogenesis in the mouse brain. Abstract and poster. International Society for Magnetic Resonance in Medicine, 17th Scientific Meeting and Exhibition. Hawaii Convention Centre, Honolulu, Hawaii, USA, April 18-24, 2009.
17. Kokaia Z. Neural stem cells for the cell therapy of stroke damaged brain. 10th International Conference on Neural Transplantation and Repair. September 10-13, 2008, Freiburg, Germany.
18. Kokaia Z. Neural stem cells and ischemic stroke. Ladislav Tauc Conferences: Synaptic Plasticity and Brain Repair. December 11-12, 2008, Gif sur Yvette, France.
19. Cusulin C, Darsalia V, Allison S, Kuzdas D, Kallur T, Monni E, Lindvall O. and Kokaia Z. Transplantation of Human Fetal Neural Stem Cells in Stroke Lesioned Striatum and their Interaction with the Local Environment. 7th Annual Meeting of the International Society for Stem Cell Research. Centre Convencions Internacional. Barcelona, Spain. July 8-11, 2009.
20. Kokaia Z. Neurogenesis in the adult brain after ischemic stroke. 9th European Meeting on Glial Cells in Health and Disease. September 8-12, 2009, Paris, France.
21. Ahlenius H, Darsalia V, Iosif R, Torper O, Jacobsen S-E, Lindvall O. and Kokaia Z. Insult specific involvement of adaptor protein Lnk on neural stem and progenitor cell proliferation. 7th Annual Meeting of the International Society for Stem Cell Research. Centre Convencions Internacional. Barcelona, Spain. July 8-11, 2009.
22. Kokaia Z. Neural stem cells and ischemic stroke. Stem Cells: Biology and Use in Vascular Brain Disorders. September 17-18, 2009, Leuven, Belgium.
23. Kokaia Z. Stem cell therapy for recovery after ischemic stroke. 4th WSO Regional Meeting: Stroke Prevention, Diagnosis and Treatment. September 24-26, 2009, Tbilisi Georgia.

The information about StemStroke consortium, its goal and work has been covered by several newspapers and news programs at Swedish Television (for details see StemStroke webpage: http://www.stemstroke.eu/mediafiles). StemStroke was also represented in the book "New Therapies-EU supported Research 2002-2006" (could be downloaded from StemStroke webpage: http://www.stemstroke.eu/mediafiles/new-therapies.pdf/view). Based on this publication, we have published a leaflet which is widely distributed by consortium members a various meetings.

Reagent availability

Several cell lines generated by StemStroke consortium Partners are available. Among them:

1.The cortical foetal human NS cell line CB660

2. The cortical foetal human NS cell line CB541

submitted to UK stem cell bank and to the BioRep repository for distribution to the scientific community.

3. NES cell line, AF22O (also expressing GFP or DsRed – AF22) most characterised, derived from dermal fibroblast derived iPS cells created using retroviral delivery of three factors, Oct4, Klf4 and Sox2.

are available for other StemStroke partners to explore.

4. Human fetal striatum and cortex-derived NSC lines (neurospheres)

5. Human fetal striatum-derived NS cell line (adherent)

are available upon request to Partner 1.

In conclusion, the work of the consortium during the whole period was carried out according to the Technical Annex. Although there were some delays and modifications of several Milestones and Deliverables (see periodic reports), there were no major hurdles and the consortium have achieved basically all goals which were planed to be achieved.