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The 2nd Step by Step International Spinal Cord Repair Meeting

—Combining research Step by Step into multi-pronged approaches for spinal cord repair

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ABSTRACT

On April 26-27, 2013, the Step by Step Foundation hosted the Second International Spinal Cord Repair Meeting at the Fira Barcelona Convention Center in Hospitalet de Llobregat, Spain, highlighting some of the exciting research including clinical trials which show promise for treatments for this devastating disorder. This meeting brought together clinicians, clinical scientists and molecular biologists from more than 10 countries to evaluate current knowledge on clinical, cellular, and biomolecular aspects of spinal cord injury. A major goal of the conference in advancing the translation of research data to the clinic was to promote multi-pronged approaches for therapy of this complex problem.

Keywords: Spinal Cord Injury; Immunomodulation; Glial Scar; Nerve Grafts; Neurotrophins; Neural Stem Cells; Electromagnetic Stimulation; HSV-1 Vectors; Cholecalciferol; Vitamin D3; Wnt Proteins; Neuroregeneration

*Conference Organizing Committee.

1. INTRODUCTION

Spinal cord injury (SCI) is a major cause of long-term disability with no clinically effective treatment, devastating the lives of those affected as well as their families. SCI is produced by multi-factorial processes as a result of primary mechanical damage, secondary cell death, reactive gliosis and a poor capacity to regenerate damaged axons. Depending on the transition from the acute to chronic phase, SCI can have varying final impact on normal sensory and motor functions. Currently the only drug to treat acute spinal cord injury is methyl-prednisolone, administered in order to prevent secondary inflammatory neural damage, but with no further reparative effect. The only help to prevent the debilitating long-term effects of severe chronic spinal cord injury come from rehabilitation programs consisting of daily exertions to maintain the injured patients in good physical and psychological condition. Finding new neuroprotective molecules for alternative and/or complementary treatment remains an important goal in this field.

Animal models have been used to test several possible therapies for this condition, including pharmacological, gene and cell therapies. Promising data indicate that a

variety of treatments including growth factors, glial scar neutralizing agents, grafts of glial and stem cells (both embryonic and adult), can promote spinal cord repair and functional recovery after a lesion. Neural stem cells (NSCs) possess the ability to self-renew and to differentiate into the three major cell types found in the central nervous system (CNS): neurons, astrocyte and oligodendrocytes. Recent studies have shown that epigenetic gene regulation events such as DNA methy lation and histone modification play important roles in regulating NSC fate specification. Therefore, by controlling differentiation it may eventually be possible to treat SCI with the appropriate neuronal and glial cell types: e.g. generation of new neuronal relays to recuperate connectivity, oligodendrocytes to combat demyelination, or differentiated (non-reactive) astrocytes to help to control glial scars.

The Step by Step (SbyS) Foundation, hosted this 2nd International Spinal Cord Repair Meeting, is a non-profit organization dedicated to the recovery of people suffering from neurological disorders of the CNS, particularly SCI and stroke. Its founder and president, Frederic Crespo, is an architect based in Barcelona who became paraplegic in a car accident in 2004. SbyS's main activity is carried out in its rehabilitation center where its physiotherapy methodology has made it one of the Europe's most successful in treatment of SCI victims. Apart from the foundation's commitment to rehabilitation, SByS has a special interest in promoting other areas of research focused on functional recovery after SCI, as monitored by its scientific committee: Miguel Ángel González Viejo (Vall d'Hebron Hospital, Barcelona), Anna Veiga (Center of Regenerative Medicine, Barcelona), Thomas Graf (Center for Genomic Regulation, Barcelona), Merce Avellanet (Hospital Nostra Senyora Meritxell, Andorra), Filip Lim and Maria Teresa Moreno-Flores. Amongst other activities SByS has now hosted two International Spinal Cord Repair meetings to bring together researchers, clinicians and patients to discuss and evaluate ongoing laboratory experiments and clinical trials with the aim of promoting interchange of experiences from the different fields, of world experts dedicated to understanding the complex problem of SCI and its treatment. http://www.fundacionsbs.org/en/ http://www.spinalcordmeeting.org/

2. SPINAL CORD REPAIR AS A FORM OF WOUND HEALING

In the first session, Michal Schwartz (Weizmann Institute of Science, Rehovot, Israel) presented her work which has led to the view that spinal cord repair can be considered as a form of wound healing in which the major problem is the lack of proper resolution of the scar. The fact that the central nervous system (CNS) is isolated from the circulation by barriers, coupled with its features as an immune privileged site, created the basis for the common view that both resident activated microglia and infiltrating immune cells are detrimental to the CNS. These properties led to the further assumption that the CNS tissue is unable to tolerate any immune activity under any conditions. Moreover, entry of immune cells to the brain was assumed to result from breakdown of the blood-brain-barrier (BBB), and even immune cell entry through other CNS barriers was considered to be the first step leading to inflammatory disease. In contrast to these assumptions that uncontrolled and detrimental monocyte invasion occur due to impaired BBB function, the data presented by Dr. Schwartz show that monocytes are required for CNS recovery following injury, and that their trafficking is orchestrated through the brain choroid plexus epithelium within the blood-CSF-barrier, by selective chemokines and integrins, which direct the type of cell recruited, and further, provide the entering cells with a controlled microenvironment. Detailed examination of the specific contribution of monocyte-derived cells to the repair process at the site of the trauma revealed that microglia are the first immune cells to be activated, yet these cells are dependent on infiltrating monocyte-derived macrophages for resolution of their function; the infiltrating monocyte-derived macrophages locally develop the phenotype of Ly6Clow/CX3CR1high, and express features of M2, resolving, macrophages. Their activity is dependent on IL-10 and MMP-13, factors that are required for resolution of the inflammation and for scar degradation. Moreover, the entire process of recovery recapitulates many aspects of wound healing outside the CNS, except that the different processes along the healing pathways are compartmentalized, to ensure their function without damage to the delicate tissue of the CNS. Together, the immune processes that accompany CNS recovery constitute a full network of the innate and adaptive immune responses occurring in the periphery. Full understanding of the immune response that mediates CNS recovery, including its similarity to healing outside the CNS and its adaptations to the unique nature of the CNS tissue, will contribute to novel therapies based on harnessing the body's own repair mechanism.

3. NERVE GRAFTS AND TROPHIC FACTORS

The participants were given some insight into state-of-the art microsurgery by Mikael Svensson (Karolinska Institutet, Stockholm, Sweden), who described their surgical advances in repairing the spinal cord with peripheral nerve grafts (PNG) and adjuvant acidic fibroblast growth factor (FGF1), a technique which has previously resulted in partial functional recovery. To aid microsurgical placement of peripheral nerve grafts, a

graft holder device was previously developed by this group and with the intent for translational development they are currently investigating a new biodegradable (calcium sulfate) graft holder device containing peripheral nerve grafts with or without FGF1. Experiments were conducted on rats subjected to a T11 spinal cord resection: subsequent repair used twelve white-to-grey matter oriented peripheral nerve grafts prepositioned in a biodegradable device with or without slow release of FGF1. Animals were evaluated with BBB-score, electrophysiology and immunohisto-chemistry including anterograde BDA tracing. At 20 weeks after grafting, the reappearance of motor evoked potentials (MEP) was observed in the lower limb and these MEP responses were further improved in the group treated with adjuvant FGF1. Reappearance of MEPs was paralleled by improved BBB scores, NF-positive fibers and anterogradely traced corticospinal fibers distal to the injury while resectioning of the spinal cord ablated signal transmission. The results continue to support the idea that some regeneration of the spinal cord may be achieved by the combination of peripheral nerve grafts and growth factors.

4. THE WNT FAMILY AS THERAPEUTIC TARGETS

Francisco Javier Rodriguez (Hospital Nacional de Parapléjicos, Toledo, Spain) summarized recent literature implicating the Wnt family of proteins as regulators of many biological events in the developing and adult CNS. He underlined their therapeutic relevance describing various strategies where modulation of canonical and non-canonical Wnt-dependent signaling pathways have produced benefits in different experimental models of CNS disorders and spinal cord lesions. Previous work by Dr. Rodriguez's group in a rat model of SCI demonstrated that trauma induces a dramatic and time-dependent change in the physiological pattern of Wnt mRNA expression, as well as the activation of the canonical pathway in cells around the wound core in a pattern suggestive of a role in glial scar formation. In particular, all Frizzled receptors were observed to be expressed in the adult spinal cord of rats and after contusion SCI, were differentially regulated and highly expressed by cells close to the wound core. Among these, detailed characterization of the Frizzled-5 (Fz5) receptor revealed up-regulation in both glial and inflammatory cells and induction in damaged axons at the injury epicenter. Ryk, a well-known non-conventional Wnt receptor was found to be expressed by macrophages, fibroblasts and astrocytes, and when inhibited by administration of a function-blocking antibody through a subdurally implanted catheter, a significant reduction of the macrophage/microglial response and sensorimotor functional improvement was observed [1].

5. A VITAMIN D CLINICAL TRIAL IN PARAPLEGIC PATIENTS

Previous work by the research group of Francois Féron (Aix Marseille University, France) showed that vitamin D exhibits immuno-modulatory, neuroprotective, antimitotic and neuroregenerative properties. In a pharmacological study, based on a rat model of peripheral nerve lesion, they observed that vitamin D3 (cholecalciferol) is more efficient than vitamin D2 and, when delivered orally at high dose (500 IU/kg/day), cholecalciferol induces dramatic locomotor and electrophysiological recovery. Vitamin D3 was also observed to increase the number of preserved or newly formed axons in the proximal end, the mean axon diameter in the distal end, neurite myelination in both the distal and proximal ends as well as the expression of genes involved in axogenesis and myelination [2]. Therapeutic effect of vitamin D on the central nervous system was also assessed. In a first study, using a rat model of spinal cord compression at the T10 thoracic level, cholecalciferol was orally delivered at 50 IU/kg/day or 200 IU/kg/day. When compared to control animals, cholecalciferol-treated rats displayed a significant improvement of ventilatory frequency and a reduction of H reflex indicating functional improvements at three months post-injury [3]. In a second study using a rat model of cervical hemisection (C2), a higher dose of oral cholecalciferol (500 IU/kg/day) was delivered weekly during 12 weeks. Improved locomotor recovery and a significantly higher rate of axons crossing the lesion site in treated animals were observed. With these convincing data in hand, and the long-established safety parameters of vitamin D in human, Dr. Féron will lead with Dr Roche a phase II multicentric, randomised, double blind clinical trial to assess the efficacy of cholecalciferol in patients with a cervical trauma. The cohort will include 60 patients with a complete injury. Patients will be treated for one year, from Day 1 post-injury, with decreasing doses of cholecalciferol. The primary outcome will be the improvement of the Upper-Extremity Motor Score (UEMS) recovery. Secondary outcome measures will include sensory index scores, spasticity, pain and quality of life.

6. TARGETED DRUG DELIVERY BY GENE TRANSFER FOR SPINAL CORD INJURY PAIN

Because of the pleiotropic effects of neurotransmitters, receptors and ion channels which can be present at multiple sites in the nervous system, the ability to use small molecules to interrupt nociceptive neurotransmission is limited to systemic administration at suboptimal

concentrations. David Fink and Marina Mata (both at the University of Michigan, Ann Arbor, USA) are addressing this problem, by using non-replicating genomic herpes simplex virus (HSV)-based vectors that efficiently target gene delivery to dorsal root ganglia (DRG) from skin inoculation. HSV-mediated production of the opioid peptides enkephalin and endomorphin-2, or glutamic acid decarboxylase (GAD) to achieve the release of gamma amino butyric acid (GABA) from DRG neurons reduces pain-related behaviors in rodent models of inflammatory pain, neuropathic pain and pain caused by cancer. In a rodent model of below-level pain after spinal cord injury created by lateral hemisection, subcutaneous inoculation of the GAD-expressing vector reduced mechanical allodynia and thermal hyperalgesia resulting from the injury. In a similar model of below level pain created by blunt trauma, subcutaneous inoculation of an HSV-based vector expressing the anti-inflammatory cytokine IL10 reduced pain measured by evoked behaviors and by a conflict-avoidance task. The Mata/Fink group has used a similar strategy of targeted delivery to enhance regeneration of ascending and descending projections after injury. Because activation of Rho GTPase serves as a final common path for the several inhibitors of axon growth, they constructed a non-replicating genomic HSV-based vector to express clostridial C3 transferase (C3t), a bacterial protein that inhibits Rho signaling by N-ADP ribosylation of Rho GTPase. Subcutaneous inoculation of the vector into the skin of the forepaw one week after a dorsal C5-T1 rhizotomy resulted in expression of C3t in dorsal root ganglion (DRG) neurons and inhibition of Rho GTPase activity, which enhanced axonal regeneration through the dorsal root entry zone into the spinal cord, correlating with improved sensory-motor coordination of the forepaw. Injection of the vector into motor cortex one week after cervical spinal cord dorsal hemisection resulted in regeneration of a substantial number of corticospinal tract fibers by four months following injury.

7. PROMOTING ACTIVITY-DEPENDENT PLASTICITY TO REINFORCE BENEFICIAL EFFECTS OF EXERCISE

After spinal cord injury (SCI), intensive training can improve locomotor function in patient and animal models of SCI. This training-induced recovery, however, is limited. For individuals with SCI rehabilitation training is extremely labor intensive and often requires the participation of several therapists to assist with leg and trunk movements. Overall, the best results of training have been seen in young animals and children which have been attributed to limited plasticity in the adult spinal cord. Diminished conduction of axons spanning the injury and below, is among major factors that limit plasticity in adult mammalian spinal cord. Victor Arvanian (Northport Veterans Affairs Medical Center and Stonybrook University, NY, USA) presented his group's work to develop an alternative set of treatments to strengthen transmission and promote plasticity in the damaged spinal cord, with the final goal of reinforcing beneficial effects of exercise on locomotor function after SCI. Several components of such treatment were considered in order to improve synaptic plasticity, axonal growth/sprouting and recovery of locomotor function after partial spinal cord injury. Major lines of research discussed were: 1) identification and neutralization of the major myelin- and scar-related inhibitory factors that block axonal conduction in damaged spinal cord; 2) provision of sufficient neurotrophin support to enhance plasticity and re-myelination; 3) activation of synaptic inputs to spinal neurons to recover activity of membrane receptors which enter into a silent state following denervation induced by injury; 4) combination of these treatments with rehabilitation exercise. For efficient and clinically relevant delivery of the treatment components the Arvanian group is using gene therapy-based approaches, such as engineered fibroblasts and viral vectors derived from adeno-associated virus (AAV). In order to neutralize the effects of NG2, a major scar-related inhibitory factor restricting the growth of fibers and axonal conduction after SCI, in collaboration with Dr. Joel Levine and the PENN Vector Core (University of Pennsylvania, USA), they have recently generated a new viral vector based on AAV-10 expressing NG2-neutralizing-antibody (AAV 10-NG2Ab). A similar AAV10 vector was also constructed for delivery of the neurotrophin NT-3. The AAV10 serotype was chosen based on previous studies showing that among the several AAV serotypes tested, AAV10 induced the best transduction of oligodendrocytes and NG2-positive cells in the damaged spinal cord. To enhance plasticity and provide muscle contraction through activation of pre-excising physiological pathways, spinal repetitive electromagnetic stimulation (EMS) was used. The effects of treatments were evaluated by electrophysiological techniques including intra-axonal, intracellular, extracellular and intramuscular recordings from the live anesthetized adult rats. In addition, multiple behavioral testing, anatomical tracing and immunochemistry were conducted. Repetitive EMS induces facilitation in a LTP-like fashion which lasts for at least 2 hours after withdrawal of the stimulation. Minipump administration of NG2-neutralizing antibodies improved nerve transmission and when exercise training was applied during the repetitive EMS-induced LTP period, locomotor function was also improved.

8. TRANSPLANTATION OF NEURAL STEM CELLS

8.1. A Clinical Trial with Human Neural Stem Cells

Armin Curt (Balgrist University Hospital, Zurich, Switzerland) reinforced the importance of electrophysiology as one of the outcome measures that are most sensitive to reveal improvements which are considered to be amendable by the specific mode of action of a therapeutic agent. Dr Curt is leading a clinical trial for SCI therapy using human neural stem cells (produced by StemCells Inc.) in patients with complete (ASIA A) to incomplete (ASIA C) lesions. So far most SCI trials apply rather nonspecific outcome measures (like ASIA scores) that although are of clinical relevance, are not able to reveal underlying mechanisms of recovery in SCI. This creates a dilemma where the considered interventions are rather specific but clinical trial protocols fail to monitor the mode of actions (both in the sense of drug/cell activity and specific consequences in body function). This leaves the field in the unfortunate situation that translational information gained in clinical trials mirrored back to basic research is rather weak and the potential reasons in failures of interventions (like complete failure or insufficient induction of drug/cell activity etc.) cannot be discerned, thus resulting in the incapacity to modify interventions for improved efficacy. In this aspect SCI research falls behind other areas in neuroscience (like MS research) where surrogate markers have been successfully introduced to overcome the above mentioned shortcomings. In addition to assessing safety, the trial in Zurich evaluates preliminary efficacy based on defined clinical endpoints, such as changes in sensation, motor function and bowel/bladder function. Importantly, quantitative tests of specific sensory function and electrophysiological measures of impulse transmission across the site of injury are conducted to determine any association with the clinical examination, thus providing further objective confirmation of functional improvements.

8.2. Neural Stem Cells in Rat SCI Models

To address the mechanisms by which neural stem cells may promote SCI repair, Armin Blesch (University Hospital Heidelberg, Germany) reported on his work in collaboration with Paul Lu and Mark Tuszynski (University of California at San Diego, USA) showing that neural stem cells (NSCs) and neuronal progenitors grafted into a rat model injured spinal cord can extend axons over large distances, serve as neuronal relays and rebuild damaged spinal circuits thereby improving functional deficits [4]. Using GFP-expressing transgenic rats, technical limitations of distinguishing donor cells and their extensions from host tissue were overcome. The data suggested that properties intrinsic to early stage neurons can overcome the inhibitory milieu of the injured adult spinal cord to reconstruct neuronal pathways for functional recovery and provided the basis for the hypothesis that not only somatomotor function but also the regulation of autonomic cardiovascular functions can be improved by newly generated neuronal relays. To pursue this goal and to further elucidate cellular mechanisms of sympathetic regulation after injury, embryonic day 14 (E14) brainstem-derived neural stem cells (BS-NSCs) from GFP transgenic rats were grafted into the lesion site of T4 complete spinal cord transections in adult wild-type rats. Control animals were either grafted with E14 spinal cord neural stem cells (SC-NSCs) or subjected to injury alone (without grafting). Eight weeks later, grafting of BS-NSCs but not SC-NSCs resulted in full recovery of resting cardiovascular parameters and alleviated colon distension-induced autonomic dysreflexia. This recovery was accompanied by graft-derived axon innervation of sympathetic preganglionic neurons caudal to the lesion site and was dependent on the innervation of grafts by host axons. Thus, differentiation of neural stem cells into appropriate phenotypes and the generation of neuronal relays may provide the opportunity to partially restore motor and autonomic dysfunction after spinal cord injury.

8.3. Neural Stem Cell Transplants in Mouse SCI Models

Kinichi Nakashima (Kyushu University, Fukuoka, Japan) next introduced his work showing that the histone deacetylase (HDAC) inhibitor and well-known anti-epileptic drug, valproic acid (VPA) enhances neuronal differentiation of neural stem cells. He proposed that since the patterns of NSC differentiation are exquisitely controlled during normal embryonic development, restoration of damaged neural networks in the injured adult CNS is severely limited due to lack of appropriate signalling. Using a mouse model of spinal cord injury (SCI), administration of VPA and transplantation of mouse NSCs were observed to enhance hindlimb functional recovery. Neuronal differentiation of transplanted NSCs was promoted in VPA-treated mice. Anterograde corticospinal tract tracing revealed that transplant-derived neurons partially reconstructed the broken neuronal circuits, most likely in a “relay” manner. Transplanted cells were derived from transgenic animals expressing diphtheria toxin receptor, thus allowing their ablation at will by adding diphtheria toxin. Ablation of the transplanted cells abolished the recovery of hindlimb motor function, indicating that these graft cells contributed directly to the improvement of motor function. These data raise the possibility that epigenetic regulation of

transplanted neural stem cells can be exploited to provide treatment for SCI. As indications of future directions of his work, Dr Nakashima also showed promising results using xenotransplantation of human NSCs derived from induced pluripotent stem cells into a SCI model in nude mice.

9. DISCUSSION AND CONCLUSIONS

This second international conference organized by the Step by Step foundation succeeded in bringing together high profile international speakers, delivering frontline news, with a mixed audience of clinicians, researchers and patients. An impressive amount of new data was shared between all participants and everyone found a good reason to remain optimistic. At the same time it was pointed out that no miracle cure was to be expected in the short term and sensationalist "breakthrough news" should be cautiously considered. As exemplified by the Foundation's logo, a step by step evolution of advances was considered the most solid and credible for such a dramatic and complex problem. The main value of the Step by Step conference was enhancing personal exchange—this was particularly successful because of the small size of the conference and the multidisciplinary nature of the speakers. By means of a roundtable discussion at the end of the sessions on each day, the researchers brought up interesting opinions of all the participants. Several key points were brought up: 1) inflammation must be controlled adequately and with correct timing, especially keeping in mind that SCI repair failure is due to the incomplete resolution of the scar. Importantly, the immune cells which most participate in this task appear to have only limited access via the choroid plexus; 2) old ideas advance with new technology-the classical combination "nerve grafts + neurotrophins", together with biodegradable scaffolds shows promise in promoting some fiber regeneration and limited functional recovery; basic research continues to reveal new potential therapeutic targets such as the Wnt family of molecules which are regulated by SCI and are highly likely to have a role in its pathophysiology; 3) therapeutic agents already approved for other clinical uses may be useful for treating SCI—for example, a phase II clinical trial is planned to test the efficacy of cholecalciferol (vitamin D3) in patients with a complete injury cervical trauma; 4) one of the greatest challenges in treating SCI is how to achieve localized drug delivery to delicate nervous tissue in a noninvasive manner-HSV-1 vectors inoculated in skin/muscle can safely achieve localized drug delivery to the human spinal cord via gene transfer; 5) the beneficial effects of exercise training on SCI can be augmented considerably if combined with the promotion of plasticity with spinal repetitive electro-magnetic stimulation (EMS) and continuous infusion of NG2-neutralizing antibodies; 6) promising results from neural stem cell grafts in different SCI animal models now indicate that electrical connectivity has been observed to be re-established between spinal cord zones rostral and caudal to the lesion and re-transection or graft cell ablation removes effect. Although caution must still be applied to interpreting these results, even the most skeptical researchers at the conference conceded that the possibility exists of new neuronal relays are formed by the graft, although further work is needed to discard other mechanisms such as fiber sparing and sprouting. Significantly, a clinical trial with neural stem cell grafts is now being conducted with an emphasis, in addition to assessing safety, on evaluating efficacy based on defined clinical endpoints, quantitative tests of specific sensory function and electrophysiological measures of impulse transmission across the site of injury.

During two days of the conference, several innovative therapeutic approaches—new drugs, biomaterials, tissue grafts, cell and gene therapy, exercise, electromagnetic stimulation—were exhaustively described. Nevertheless, everyone was realistic in defining the investigation of his/her proposed strategy to a specific subset of SCI: e.g. acute vs chronic, complete vs incomplete, or motor vs sensory. As a general consensus, it was predicted that future efficient therapies will include several treatments, probably delivered in a sequential manner. A concern was expressed by patients that relatively little research was being done using chronic models of SCI. Although clinicians, researchers and patients urge for effective treatments as soon as possible, it is unlikely that initial therapies will provide a complete cure for such a complex disorder. Thus it is essential that all clinical trials be designed and conducted with as much interchange of information as possible with laboratory researchers since advances in therapeutic effectiveness will depend on increasing understanding of the cellular and molecular mechanisms underlying SCI. At the end of the conference, organizers and contributors agreed that a third international Step by Step meeting should be organized, possibly with other international foundations.

10. POSTER PRESENTATIONS

10.1. Moving iPS Cell Technology Closer to the Clinic: Making HLA Matched Clinical Grade iPS Cells from Human Cord Blood Using Modified RNA Transfection Methods

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The Barcelona-based Pluripotency Laboratory is advancing induced pluripotent stem cell (iPS cell) technology closer to ground state pluripotency for clinical

applications and is part of the University of Barcelona Faculty of Medicine. We aim to understand the role of cell cycle and epigenetic genes in attaining ground state pluripotency. Furthermore we aim to define efficient differentiation protocols of iPS towards progenitor stem cells of various tissues. The laboratory addresses a number of major bottlenecks in the field such as the threat of genetic instability, immune response of iPS-derived cells and to define clinical cell culture conditions for eventual cell replacement therapy for different types of human disease. The work will focus on developing the technology to make high quality clinical grade iPS cells that are HLA-matched to the Spanish population. Here we highlight the current stage in differentiation of iPS cells towards cells to treat spinal cord injury. For more information please see the laboratory web page.
http://pluripotencylaboratory.wordpress.com/

10.2. Human Organotypic Slice Culture as *in Vitro* Model of Spinal Cord Injury to Study Allogeneic Neural Cell Therapy

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Stem cells are a promising source in the study and development of neural cell therapy for central nervous system trauma and diseases, including spinal cord injury (SCI). However, we lack a model to study allogeneic human neural cell therapy to evaluate the interaction of human donor and host neural tissue. Our aim is to develop models of SCI to evaluate human donor neural progenitor cells (hNPCs) derived from induced pluripotent stem cells and fetal nervous system in a human organotypic slice culture system. We established explants from human fetal first trimester spinal cord. The cultures were compared with *in situ* spinal cord tissue to evaluate the impact of culture *per se*. At 7 days in culture, contusion injuries were achieved in the slices by the use of the Infinitive Horizon spinal cord impactor (25 kdyne force), and 1 hour later hNPCs were grafted or sham injections were performed. 7 and 14 days later, slices with and without lesions and grafts were analyzed by immunohistochemistry and flow cytometry to evaluate tissue architecture and cell therapy potential. The human spinal cord slices presented relatively stable features 7 to 21 days in unlesioned cultures, based on the expression of markers for cell proliferation (Ki67), progenitor cells (nestin), neurons (microtubule-associated protein 2, MAP-2), astrocytes (glial fibrillary acidic protein, GFAP), microglia (Iba 1 and HLA-DR$^+$/CD11b$^+$/CD45^{low}), caspase 3 (apoptosis), and Ca^+ signaling (Oregon Green Bapta-1 AM). At 7 and 14 days post injury and/or cell graft, we observed an increase in number of activated Iba1$^+$ and GFAP$^+$ cells in response to SCI but also to hNPC grafts compared to the control.

We conclude that this novel model of human allogeneic neural cell therapy in slice cultures represents a valuable platform to study host donor interactions, in the evaluation and development of neural cell therapies in SCI.

10.3. Studies of the Immunogenicity and Immunomodulatory Potentials of Human Neural Cells of Relevance for Spinal Cord Injury Repair

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Human neural progenitor cells (hNPCs) derived from either human embryonic stem cells (hESCs) or fetal origin have potential to at least partly rescue and repair damaged spinal cord tissues. However, to develop effective cell therapies for spinal cord injury with hNPCs as donor cells, further understanding of the complex interaction between neural and immune cells would be valuable. The risk of an immune response and graft rejection must be considered while the ability of transplanted NPCs to counteract deleterious inflammatory processes should be supported. We hypothesize that neuroprotection may, at least in part, be exerted via immunomodulation by hNPCs. Here, we studied the immunogenicity and immunomodulatory potential of hNPCs cultured under equivalent conditions after derivation from human embryonic stem cells (hESC-NPCs) or human fetal spinal cord tissue (hfNPCs). The expression patterns of the immune proteins human leukocyte antigen, co-stimulatory and adhesion molecules in hESC-NPCs and hfNPCs were relatively similar with and without inflammatory cytokines present. Also, both hNPCs presented the ability to counteract an alloreaction between incompatible human peripheral blood mononu-

clear cells (PBMCs) and could up-regulate regulatory ($CD4^+CD25^+$ forkhead box $P3^+$, FOXP3) T cells. However, unstimulated hfNPCs secreted more transforming growth factor-β1 and β2 but a similar level of interleukin-10 compared to hESC-NPCs. However, hESC-NPCs but not hfNPCs displayed dose dependence in triggering PBMC proliferation, which at least partly may be due to differences in TGF-β signaling. To conclude, hESC-NPCs and hf NPCs displayed similarities but also significant differences in their immunocompetence and interaction with allogeneic peripheral blood cells (PBMCs), differences that may be crucial for the outcome of neural cell therapy after a spinal cord injury.

10.4. Cell and Gene Therapy Approaches for Regeneration of Spinal Cord Neurons

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Although animal models of spinal cord injury (SCI) are necessary for determining the relevance of a particular therapeutic strategy for spinal cord repair, for rapid screening of large numbers of factors, culture models that recapitulate key aspects of neuronal changes in SCI are needed to improve understanding of the pathological and regenerative mechanisms involved, as well as to accelerate the screening of potential therapeutic agents. We have differentiated adherent cultures of dissociated human fetal spinal cord neural precursors into postmitotic neurons which we can then detach from culture plates and successfully freeze down in a viable state. When these ready-to-use human spinal cord neurons are replated, they remain viable but postmitotic, and regrowth of their neurites can be used to test for potential neuroregenerative agents. We are developing this model to investigate therapeutic strategies such as cell therapy with olfactory mucosa cells (OMC), and gene therapy using vectors derived from Herpes Simplex Virus Type 1 (HSV-1). We have observed that coculture with OMC increases the number of neurons able to extend neurites, which are also longer than when neurites are plated alone. In parallel, we are developing viral vectors to test the effect of specific genes in our neuronal cultures. In particular, HSV-1 mutants deleted simultaneously for the immediate-early genes encoding ICP4 and ICP27 demonstrate negligible cytotoxicity in rodent models and have already been used in human clinical trials. In spite of HSV-1 vectors being relatively nontoxic in vivo and in certain cultured cell lines, cultured neurons appear to be more sensitive to cytotoxic effects of some HSV-1 mutants. We are making HSV-1 recombinant mutants with further deletions to accommodate larger transgenes (up to 30 kb) as well as to eliminate residual cytotoxicity. These will be tested in our human spinal cord neuron cultures which will also serve as a model to test for biotoxicity.

10.5. Characterization of Molecules Implicated in the Axonal Regeneration Induced by Immortalized Human Olfactory Ensheathing Glia

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Olfactory ensheathing glial cells (OEGs) have properties to facilitate the regeneration of adult axotomized neurons. However, the molecular mechanisms involved in this event are still incompletely understood. The aim of this study was to identify proteins involved in adult axonal regeneration induced by OEGs. In previous microarray analyses, we compared the gene expression profile of three rat OEG populations with different adult axonal regeneration capacity. Among analyzed genes, it was found that plasminogen activator inhibitor-1 (PAI-1), proteinase-activated receptor-1 (PAR-1), neural cell adhesion molecule (NCAM), chemokine C-X-C motif ligand 12 (CXCL12) and interleukin 1 receptor like 1(IL1rl1) are candidates for promoting axonal regeneration. On the other hand, thrombomodulin and integrin beta 4 and secreted frizzled related protein 4 (SFRP4) appeared to be implicated in the inhibition of adult axon outgrowth. We validated in a functional assay the involvement of these proteins in promoting axonal regeneration by human immortalized OEGs. In coculture, the effect of silencing expression of these proteins in human OEG monolayers on axonal regeneration of adult retinal neurons was analyzed by quantifying two different parameters: the percentage of neurons that were able to regenerate their axon, and the mean axonal lengths. Our results verified the role in promoting axonal regeneration of both PAI-1 as well as PAR-1, confirming the preliminary data obtained in microarrays and RT-PCR analysis. Additionally, we have confirmed the participation of thrombomodulin in impeding axonal regeneration. These molecules could be important in regulating adult axonal regeneration by OEGs.

10.6. CLMA Scaffold and FM19G11 for a Proper Spinal Cord-Derived Neural Progenitor Niche

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Spinal cord injury (SCI) is a major cause of paralysis. Currently, there are no effective therapies to reverse this disabling condition. Recently we have shown that acute transplantation of ependymal stem/progenitor cells (epSPC)—spinal cord-derived neural precursors-rescue lost neurological function after SCI in rodents. However, in a chronic scenario, with the axon-repulsive reactive scar, a combinatorial approach is necessary. The integration of biomaterial scaffolds for a physical support with the cell-replacement therapy and pharmacological treatments to protect and induce neuronal survival offers a good formula for chronic SCI regeneration. Here we show a new application of caprolactone 2-(methacryloyloxy)-ethyl ester (CLMA) for porous scaffolds. The epSPC can grow and expand into the scaffolds in the presence of EGF and FGF: however, significant reduction of the cell population was observed after 6 days *in vitro* (DIV). FM19G11, first described as a HIFα protein inhibitor, is able to allow progenitor cells to differentiate under hypoxia, but under normoxic conditions induces self-renewal. In the presence of FM19G11, epSPC grown in CLMA scaffolds divide significantly more at 6 DIV those treated with vehicle alone. Increased oligodendrocyte precursor marker expression is also observed after FM19G11 treatment. Overall, epSPC seeded in CLMA scaffolds and activated by FM19G11 show potential as a good combination treatment of chronic SCI.

10.7. Connexins in Neural Precursor Cells Reprogramming

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Connexins (Cx) that typically form cell-to-cell channels mediating direct intercellular communication allow cells to exchange ions and other molecules across the plasma membrane. Recently, Cxs have also been implicated in the control of the proliferation and differentiation of progenitor cells. Here we explore Cx expression and the biological implications in neural precursor cells (epSPC) derived from adult rat spinal cord. When transplanted after spinal cord injury (SCI), epSPC significantly improve functional locomotor regeneration. Moreover, FM19G11, a new chemical entity, intrathecally administrated *in vivo*, favors epSPC activation with improved functional locomotor recovery in a rat SCI model. FM19G11 and the injury-related process itself induce Cx50 and 43 gene and protein expression *in vivo* and *in vitro* of epSPC cultures. When epSPC are obtained after SCI (epSPCi) enhanced proliferative rates and better yields of oligodendrocyte-directed differentiation are obtained when compared to epSPC from uninjured donors, indicating some form of "activation" by the SCI process. A relationship of Cx50 with self-renewal and differentiation potential of epSPC activated either by the injury (epSPCi) or FM19G11 treatment was explored. Experiments using siRNA to specifically knock down Cx50 confirm direct regulation of stemness cellular markers such as Sox2 and Oct4 in undifferentiating cell growth conditions. Interestingly, both reprogramming processes, the induction of self-renewal and directed differentiation, involved Cx50 intervention. The expression of Cx43 and Cx50 during the directed differentiation of epSPCi to oligodendrocytes exhibited an inverse tendency relationship with respect to expression levels, with fast and consistent changes of subcellular location during the cell maturation process. Our observations *in vitro* and *in vivo* indicate that Cxs are involved in the self-renewal and differentiation of neuronal progenitors.

10.8. FM19G11 Favours Spinal Cord Injury Regeneration and Stem Cell Self-Renew of Spinal Cord-Derived Progenitor Cells

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We recently showed that transplantation of activated ependymal stem/progenitor cell (epSPC) can rescue lost neurological function after spinal cord injury (SCI) in rodents. In the acute phase there is a massive loss of axons, and cell bodies of neurons and glia in the lesion epicenter provoked by apoptosis and necrosis that extends to a secondary phase, affecting all populations of neurons and glia including oligodendrocytes. Furthermore, the vascular loss creates a hypoxic environment which obstructs cell self-renewal. Induction of the self-renewal and proliferation of endogenous regenerative machinery with noninvasive and nontoxic therapies could complement or even substitute cell transplantation for SCI patients. FM19G11 is a new chemical en-

tity, first described as a HIFα protein inhibitor, which enables progenitor cells to differentiate under hypoxia. Here we show the effect of FM19G11 on epSPC *in vitro* and *in vivo* when transplanted after SCI. First, in epSPC grown under normoxia, FM19G11 induced self-renewal and upregulation of stemness-related genes by inducing insulin-like signalling pathways (AKT/mTOR and AMPK signalling), probably due to intervention of mitochondrial activity. Interestingly, intrathecal administration of this compound in a sustained way immediately after SCI significantly improved locomotor activeity, with noticeable improvement from one week after lesion, compared to vehicle-treated animals. Moreover, an additive effect was obtained when epSPCi were transplanted and FM-19G11 treatment applied immediately after lesion. The increase of the stem cell population and/or differentiation caused by FM19G11 could contribute to the observed repair of tissue damage and improved locomotor recovery. Quantitative analysis revealed significantly smaller cyst size and more preserved tissue upon combined drug and cell treatment. These data point to FM19G11 as a potential drug candidate for the treatment of SCI.

10.9. Motor Assessment of Syringomyelia Model in Rats

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Posttraumatic syringomyelia is characterized by intraspinal syrinx formation often associated with progresssive neurological loss and pain. The standard treatment is to drain the cyst with a shunt tube, sometimes combined with untethering. However, the surgical treatment is often ineffective. To study mechanisms of syrinx formation and develop more effective treatments, animal models are needed, with methods to study functional aspects. These methods are however missing, and functional assessment of syringomyelia has not been performed previously in experimental research. Syringomyelia preferentially affects grey matter, which should have consequences for the synchronization of lumbar and cervical central pattern generators, and alternation between limb movements. We used rat models of thoracic syringomyelia and investigated the consistency of different functional parameters, how they correlate, and how they change over time after injury. Motor functions of the trunk, fore and hindlimbs during swimming were extensively analyzed with the purpose of establishing better methods of motor assessment. Particular emphasis was placed on the ability of the tests to differentiate between pure traumatic injuries, and those associated with intraspinal cysts. We found that the BBB-score, a standard method for evaluating animal models of traumatic spinal cord injuries, was suboptimal with respect to assessing effects of cysts. A 7-parameter swim-score and a beam-walk test showed superior results in the syringomyelia models studied. Correlation of the functional data with loss of grey and white matter, size and location of the cysts are used to analyze the morphological correlates to the functional effects of the spinal cord injuries. These methods should be very valuable in the development of therapies for this severe consequence of spinal cord injuries.

10.10. The Use of Poly (N-[2-hydroxypropyl] methacrylamide) Hydrogel to Repair a T10 Spinal Cord Hemisection in Rat: A Behavioral, Electrophysiological and Anatomical Examination

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In spite of numerous attempts to reverse the deficits due to spinal cord injury, no efficient treatment is available yet. This unfortunate fact is in part due to the development of a dense glial scar around the injury site which hinders regrowth of axotomized neurons. Glial scar formation can be limited by the implantation of a porous hydrogel fitting closely to the lesion site.

The aim of the present study was to evaluate functional recovery after T10 thoracic hemisection and acute implantation of a hydrogel block of PHPMA [poly N-(2-Hydroxypropyl) methacrylamide] in rat. This biocompatible hydrogel is already known to support axonal regrowth, neural migration and proliferation. Locomotor behaviour was analyzed weekly in an open-field during 14 weeks by the use of the BBB test. At week 15 the

sub-lesional reflexivity and the ventila tory frequency induced by evoked muscular fatigue were evaluated. Post mortem, spinal cords were extracted, fixed and marked with fluoromyelin™ and anti-NF-H antibody. This study highlights the hydrogel therapeutic effects through multiple approaches: 1) improvement of locomotor function; 2) efficient adjustment of the ventilatory function during electrically induced-isometric contractions; 3) similar H-reflex to control values; 4) NF-H positive axons extending into the hydrogel and better myelin preservation rostrally to the lesion site. Our study confirms that PHPMA hydrogel could be used as part of a reparative spinal cord therapy.

10.11. Phenotype Interconversion in Human Neural Stem and Progenitor Cells

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A number of cell types have been suggested as potentially useful for cell therapy in spinal cord injured patients. We study human fetal neural precursor cells (NPCs) to establish a treatment that can be translated into clinical practice. We have previously shown functional improvement after acute-subacute transplantation of human spinal cord-derived NPCs. To evaluate phenotype in neural cells with the purpose of isolating subpopulations, we have assayed 25 NPC lines for ten cell surface antigens. To further study the functional relevance of these markers, we have used some of them—*i.e.* CD15, CD133, CD29, A2B5 and PSA-NCAM—for fluorescence-activated cell sorting, evaluating the stability of marker expression and phenotype of the sorted cells during continued culture and after *in vitro* differentiation. Unexpectedly, for all antigens tested, and for all cases studied, the proportion of cells expressing each antigen rapidly returned to levels seen prior to sorting, implying that phenotypic identity, as displayed by cell surface antigen expression, is an unstable characteristic of cells cultivated *in vitro*. The molecular mechanisms remain to be elucidated, but may involve influence of exogenous mitogens and epigenetic factors, some of which are currently being investigated. We are also currently comparing the transcription profiles of different populations of sorted cells with their post-sorting cultured counterparts to determine if the re-setting of surface marker expression is accompanied by a similar re-setting of the transcriptome. This would imply full phenotypic intercon version. We hypothezise that any isolated subpopulation of neural precursor cells with time *in vitro*, due to continuous interconversion, attains a default marker expression pattern, implying that markers for lineage-restricted neural precursors *in vivo* cannot be used for sorting out stable populations of committed precursors *in vitro*.

10.12. Plasticity in Lumbar Segments after Spinal Cord Injury: Disinhibition, Hyperreflexia and Neuropathic Pain

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In this work we use thoracic spinal cord injuries of different severities to assess the plasticity that takes place in lumbar segments three months after injury. For this reason we studied three main points of the system, simplified here as the entrance of the stimulus into the spinal cord, the later modulation once in the spinal cord, and the elaboration of the output response. The elements taken into consideration were the GABA synthesizing enzyme (GAD65/67), the peptidergic and non peptidergic afferences, the astroglial and microglial cells, the serotoninergic innervation, and the inhibitory synapses in motoneurons mediated by gephyrin. The analyses revealed that there is an increase in the afferent input as well as an increase in the presence of GAD65/67 in the dorsal horn. A persistent gliosis was evident in both ventral and dorsal horns, and together with the loss of descending serotoninergic inhibition, confers an extra excitatory component to the nociceptive spinal modulation. On the contrary, the presence of inhibitory synapses on motoneurons was not reduced after injury. Electromyographic studies performed in the same animals demonstrate the fully functionality of the peripheral nerve and the muscle, but indicate the existence of hyperreflexia and central hyperexcitability (measured in form of wind-up responses). To sum up, and despite the intended overcompensation of the inhibitory elements, all the changes finally led to a persistent spinal hyperexcitability. These results indicate the important plastic changes that take place in lumbar segments, in order to reorganize the system after a central injury. Nevertheless, the spinal cord is immersed in such a general disinhibition process that the balance between excitation and inhibition is lost, and the excitatory component becomes predominant. At the end, all these changes promote the

appearance of hyperreflexia and neuropathic pain below the lesion site three months after the spinal cord injury.

10.13. Role of Lysophosphatidic Acid in the Pathophysiology of Spinal Cord Injury

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The inflammatory response that occurs after spinal cord injury (SCI) strongly contributes to secondary injury. However, little is known regarding the molecules that trigger this inflammatory response. Lysophosphatidic acid (LPA) is an extracellular lipid mediator with many physiological functions. Several studies suggest that LPA could trigger inflammation. We demonstrate that LPA levels are increased in the spinal cord parenchyma after SCI. Since LPA is found in serum in high concentrations, serum leakage that occurs after SCI can lead to neural cells being exposed to substantial levels of LPA. However, our results also reveal that the mRNA levels of the enzymes involved in LPA synthesis are up-regulated in the injured spinal cord, suggesting that LPA levels increase after SCI due to both, plasma extravasation and to its synthesis. In order to assess the potential contribution of LPA in SCI, we injected LPA in the dorsal column of intact spinal cord. LPA led to macrophage recruitment and demyelination, suggesting that the increase in LPA levels observed after SCI may contribute to secondary damage. LPA may mediate its effects by signaling via 6 G-protein coupled receptors (LPAR). Interestingly, intraspinal injection of LPA into the intact spinal cord of LPAR1 null mice led to reduced macrophage recruitment and myelin loss. Moreover, oral administration of AM095, a potent and selective antagonist for LPAR1, reduced functional deficits and demyelination after SCI. Overall, these results demonstrate that LPA levels are increased after spinal cord injury and contribute to secondary damage and functional deficits by signaling via LPAR1.

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