Background

Traumatic brain injury (TBI) can lead to necrosis, apoptosis and autophagic cell death of neurons following by multiple events such as opening of the blood-brain or blood-spinal cord barrier, inflammation, edema, ecotoxicity, increase in free-radicals, altered cell signaling and gene expression The microenvironment of the injured brain includes different cell types such as normal and dysfunctional neurons (surviving bodies and dead axons or synapses), denervated intact neurons, senescent cells, blood capillaries, glial scars, free-radicals, neuroinflammatory and neurotrophic factors, and the extra-cellular matric. Thus, the repair and reconstruction of the brain tissue is the ultimate goal for the treatment of brain injury. It has been observed that after injury, the brain may be attempting to repair itself, endogenous neural stem cells (NSCs) can migrate from the subventricular zone (SVZ), dentate gyrus (DG), and striatum to the injury site where they differentiate into neurons, oligodendrocytes, and astrocytes. Axon formation is a critical step to neural connection and synaptogenesis. Axon can grow and sprout from new neurons derived from NSCs. However, the spontaneous regenerative process is not sufficient for complete reconstruction and full recovery of the neuronal function (and in general in adult brain, regeneration and migration of new neurons is limited).

Also, as part of the acute injury response, glial cells (astrocytes and microglia) migrate to the injury site where they form the reactive glial scars to repair BBB, SCB and isolate the site of injury and helps in the healing process. However, the same cells start producing inhibiting growth factors limiting axonal regeneration.

Therefore, the design of neural injury response strategy needs to promote favorable factors and simultaneously inhibit adverse factors.

Cell transplantation therapy has great potential and, following cell delivery using intravenous and intraarterial injections after TBI, significant neurological improvements are observed. However transplanting stem cells in-vivo is a complex and difficult operation: 1) these cells have low capacity of migration and tend to form clusters close to the targeted site and 2) they have to integrate into the host neuronal network and survive; overall this process of migration, differentiation and integration has yet to be fully understood.

Direct cell reprogramming, or transdifferentiation, is the conversion, using typically defined transcription factors (TFs), of a somatic cell into another without inducing pluripotency. For both techniques, the main concerns are risk of tumor formation, and spread of foreign new derived neurons in unintended sites, however cell transplantation is a more involved process with a risk of triggering an immune-rejection from the host and comes with a high-cost involved in clinical grade cell production for transplantation. Yet direct reprogramming in-vivo still faces difficulties which need to be addressed:

1) Careful selection of the target cell for reprogramming to avoid depletion of the cells being reprogrammed: cells to be reprogrammed need to be carefully selected as not to impair their functional role.
2) Identify transcription factors (TFs) which promote conversion to identified neuronal cells.
3) Design of a precise and safe gene delivery system.

In the study we will address each of these points.

Design of an efficient and specific strategy which promotes reprogramming of astrocytes to neuroblasts in the adult brain after TBI with a low-risk gene delivery system.

Wenze Niu et al., in their study, selected astrocytes for cell reprogramming since they are one of the most abundant non-neuronal cells in the CNS. And following a TBI, they aggregate to form glial scars which physically and chemically obstruct axonal regeneration. Among the eight TFs (SCLA1, BRN2, KLF4, MYC, MYT1L, OCT4, SOX2 and ZFP521) and the four microRNAs (miR9, miR124, miR125 and miR128), they found that SOX2, by itself, induces the most significant number of DCX^+ cells, adult neuroblast cells (iANBs). Next, they crossed various transgenic mice with reporter-line mice, and subsequently injected them with SOX2-expressing lentivirus. They showed that 1) iANBs were only localized in the injected striatal regions and were not the result of any migration of DCX^+ cells coming from areas surrounding the injection site like the lateral ventricle, 2) the iANBs originated from resident astrocytes, and 3) the iANBs originated from the cells transduced by lentivirus under hGFAP promoter and not from $IBA1^+$ microglia or N2-glia. Additionally, they also proved that SOX-2 induced iANBS did not originate from neurons going through dedifferentiation and becoming DCX^+ neuroblasts. Then they investigated iANBs characteristics demonstrating that the neuroblasts derived from non-dividing astrocytes and passed through a proliferating state. Finally, after co-injecting BNDF-Nog-expressing lentivirus with SOX2-lentivirus, the researchers performed a whole-cell patch-clamp which exhibited mature neurons with full electrophysiological function. In the course of the experiments, they did not observe any tumor formations confirming that the newly converted neurons have been successfully integrated into the local neuronal network without immediate side-effects.

Research from Nui et al., identified SOX2 as a critical transcription factor in the induction of resident astrocytes to neuroblasts, in conjunction with the neurotrophic factor BNDF and NOG gene for induction of neuroblasts to mature neurons. We will also target NeuroD1 as Guo et Al. showed that NeuroD1 expression reprograms reactive astrocytes and N2 cells directly into fully functional neurons (glutamatergic and GABAergic neurons) without them going through a neuroprogenitor stage. We express these genes using CRISPR-Cas9 gene editing technique which is directed to a DNA sequence by a single guide (gRNA). To target each gene, we inject using AAV-sgRNA, a gRNA per gene to express, with repeats if needed [1]. For our study, we use the improved dCas9-fused activator SunTag-p65-HSF1 (SPH) and we use the SPH; GFAP-Cre double-transgenic mice to induce SPH activator in astrocytes. The TFs are activated in primary SPH astrocytes after transfection of plasmids expressing Cre and sgRNAs.
Non-viral mRNA delivery systems have recently show promising results with biodegradable ionizable lipid nanoparticles (LNPs) encapsulating mRNA (refs); however, since lentivirus and adeno-associated viruses (AAV) have repetitively successfully been used to deliver CRISPR/Cas9 to modify cells both in vitro and in-vivo, we use them as delivery system.

For brain trauma animal model, we use controlled cortical impact injury (CCI) which allows fine control of the impact (time, velocity, depth) and lateral fluid percussion injury animal (LFPI) models.
The reasons to use two TBI models, is to study cell reprograming in regard to the type of injury: rigid with skull fracture, and mild-repetitive injuries simulating concussions like the ones observed in some sports and we also use different age-groups (it has been established that brain injuries in young and older people are more fatal
For this purpose, we will have two experimental groups: strong concussions using CCI (SCONC), mild concussion using LFPI (MCONC) and within each group: young, mid-age, and older mice. Since we want also to evaluate the impact of the transcription factors DOX2 and NeuroD1, we stratify our mice in 4 groups, MCONC-SOX2, MCONC-NeuroD1, SCONC-SOX2, SCONC-NeuroD1 and within each group, we have as stated above mice from 3 different age-groups.

After TBI, we wait at least five weeks for recovery leaving enough time for astrocytes, and N2 cells to be activated and proliferate in the injury site. After CCI or LPI, first, as a control, we inject into the SPH;GFAP-Cre mouse cortex, a retrovirus expressing mCherry under GFAP promoter (AAV-GFAP-mCherry). Brain-stained sections should reveal GFAP-positive reactive astrocytes within the injury site (red). Whereas using doublecortin DCX marker, should not reveal any DCX^+ cells in the same area. Then in the experiment group of mice MCONC-NeuroD1 and SCONC-NeuroD1 we inject AAV-sgRNAs targeting NeuroD1 on the right side of dorsal midbrain close to the injury site. To further confirm that the inductions of neurons is independent of migration of neuronal stem cells (NSCs) from endogenous neurogenic sites (SVZ, DG, striatum) we also inject on the contralateral side of the injection AAV-GFP-mCherry. We analyze brain sections close the injection site and contralateral side. After staining with the neuronal marker NeuN, immunofluorescent images for the different regions should show mCherry(red) and NeuN (green) expressions and only in the injected sites these two expressions are co-localized, and there should be a higher number of cells GFAP-mCherry + sgRNA cells compare to GFAP-mCherry cells.

[1] In past studies, expression of a single gRNA is not sufficient to induce gene expression and co-transfection up to four gRNAs per gene has led to synergistic effects with an increase of gene expression.

To confirm that the induced neurons are indeed newly generated, we have isolated few mice from each group, MCONC-NeuroD1 and SCONC-NeuroD1, and for four weeks after injection of AAV-sRNA (NeurD1), we continuously add BrdU to drinking water before immunostaining. We should expect Brdu (red) cells co-localized with NeuN (green) cells.
To further characterize the converted NeuroD1 neurons, we perform immunostaining with VGluT1 (glutamergic neurons) and GAD67(GABAergic neurons) antibodies, and to analyze how deep these neurons are located, we also use superficial and deep layer cortical markers: Cux1, Lhx2 (superficial markers) and Ctip2, Otx1, Tbr1 (deep layer markers). We expect to see the NeurD1-converted neurons to be positive for the deep layer markers.
A week after AAV injection, we perform whole-cell-patch-clamp recordings on mature neurons selected using morphology and mCherry expression in the injected region of live brain slices to examine their electrophysiological properties. We expect to be able to generate action potentials in response to step injection of depolarizing current in current-clamp mode. In addition, the same cells should display spontaneous postsynaptic currents in voltage-clamp model indicating that these neurons have formed functional synapses and have functionally integrated into the local neuronal network. We reiterate a similar experiment with MCONC-SOX2, and SCONC-SOX2 mice using plasmids expressing Cre and sgRNAs targeting SOX2, BNDF and Nog genes.

MRI is non-evasive technique of repeated imaging measurements to detect structural and functional integrity of the brain allowing tracking the temporal trajectory of the brain injury. Before and immediately after the injury, mice are subject to different behavioral tests and the loss-of-righting-reflex (LRR) time is measured. 30 minutes after recovery, the Neuro Severity Score (NSS) is measured for each group of mice. At day 2, day 7, day 14, day 30, day 60, NSS is measured again and the mice undergo the same initial tests and MRI scan.
For behavioral tests, mice are tested for 1) anxiety-like behavior using an elevated-plus maze 2) spatial cognition with a water maze, 3) locomotor-social behaviors using a circular open area testing behavioral interactions 4) sensorimotor ability using a narrow wooden beam 5) depression-like behavior using the forced swim test

https://www-ncbi-nlm-nih-gov.proxy1.library.jhu.edu/books/NBK299210/#ch42_sec2

https://www.vectorbiolabs.com/product/vb2701-aav-with-cag-promoter-driven-hchr2h134r-mcherry/
AAV=CAG-hChR2-mCherry

Construct of CRY2-SPH
oFMRI
list of TFs