BT5130 Tissue Engineering - REPORT 4

Neural Tissue Engineering: Designing constructs for better emulation

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I Introduction - Origin of proposal

The central nervous system, that consists of the brain and the spinal cord, has a very sophisticated architecture in terms of connectivity and complexity (1). This makes Neural Tissue Engineering (NTE) very complicated and difficult. Also, the regenerative ability of neurons is very less compared to other cells in our body. Consequently, the brain's capacity to self-heal in conditions of injury is very low, thus emphasizing on the need for novel NTE approaches which solves the problem at hand, without causing additional problems (2).

In case of injuries, autologous grafts are always preferred over allogeneic and xenogeneic grafts, owing to immune response, but the supply is however limited. Hence, the need for a better engineering principle along with the complexity involved in brain tissues makes it even more special and important to provide a safe and effective transplant which supports better emulation. One such scenario is the **traumatic brian injury (TBI)**, which is a non-degenerative and non-congenital distortion to the brain which is caused by some sort of an external mechanical force. **Diffuse Axonal Injury (DAI)** (3) is a type of TBI that is caused due to sudden, external and physical assault to the brain. DAI can be characterized into three grades based on histopathology and MRI (4), and this solution aims to address grade-1 damage which shows microscopic evidence of axonal damage in the white matter at corpus callosum. Since the axons are damaged, the proposed idea is to place the scaffold of cells at a position connecting the prefrontal cortex and the grey-white junction, so that neurons can regenerate and re-establish their connections.

II Review of status of research and development in the subject

Scaffolding - ***Electrospun scaffolds*** offer direct control over physical, chemical and mechanical properties of the material, thus enabling fine-tunability in the porosity and architectural norms (5).

Reports suggest that composite polymers such as PCL/PLGA and PLLA/collagen polymers are commonly used as they bring together materials that mimic different characteristics of the extracellular matrix (ECM) better (6). Spider silk (SS) is a potential candidate at being the ideal biomaterial for scaffolding, as it is both strong and extendible (7). Human primary fibroblasts, platelet-rich plasma and a variety of other stem cells have been engineered to successfully adhere to recombinant SS matrices (4RepCT) (8). However, transplantation of these scaffolds *in vivo* can cause secondary injuries that might worsen the condition (9). Several axon directing conduits and nerve grafts are established (10) (11), but their performance in the CNS, especially spanning the grey-white junction is not widely tested.

Hydrogels are commonly preferred owing to their soft texture, high flexibility and other characteristics as they resemble the *in vivo* environment well (12) (13). Various studies have used Hyaluronic acid (HA)-based hydrogel, both *in vitro* and *in vivo* (14), employing a diverse set of cells such as NPCs, ReNcells, AT-MSCs and NSCs, due to their innate properties of biocompatibility and biodegradability (15). Laminin is another material that is widely used for NTE, although they are known to elicit effects that hinder axonal growth and regeneration (16). Hence in this study, we stick to HA hydrogels. A recent development in the field has brought in ***injectable hydrogels*** (17) that can be transplanted to the site of injury via a minimally invasive surgery, which enters like a fluid, but reaches sol state upon injection and holds the cells in position. Injectable hydrogels require *in situ* crosslinking which can be ensured in multiple ways. The required linkage of the hydrogel could be via a Schiff's base linkage, which bridges the aldehyde groups in HA hydrogels and amino groups in N, O, carboxymethyl chitosan (18), or by attaching small moieties such as tetrazine and TCO to the HA hydrogel and use them as a heterogel to form a click-crosslinked hydrogel *in vivo* (19). A recent article (20) reports an amide reaction between HA and cystamine dihydrochloride catalyzed by EDC and NHS[1] was used to form a chemically crosslinked hydrogel. The EDC and NHS generate CO_2 bubbles, which due to the short gelation time, are not allowed to escape, but remain trapped inside the structure to form a porous microenvironment.

[1] 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS)

Cells - ***Neural stem cells (NSCs) and Neuronal Progenitor cells (NPCs)*** are the most commonly used cell types that are easily differentiated into neurons when constituted with common neurotrophic factors in Neurobasal or DMEM medium (2).

Mesenchymal stem cells (bone-derives and adipose tissue-derived cells) have immunomodulatory properties, produce various growth factors and also differentiate into multiple cell lineages (including neurons and similar cells). Hence they have been widely used in NTE applications (21).

Embryonic stem cells and iPSCs are known for their striking ability to easily self-renew and differentiate into any kind of cell state, compared to other methods. Although, there is a possibility of uncontrolled proliferation which might lead to teratoma formation, i.e., cancer. To ensure that it doesn't happen, Willerth et al (22), modified the cell line to carry an apoptotic gene which is expressed specifically in undifferentiated cells.

Human Umbilical cord-mesenchymal stem cells (hUMSCs) are a special kind of cells that can be differentiated into cell lineages belonging to any germ layer, are easy to access and handle, are advantageous due to their pluripotency, extracted via a painless procedure on donors and lack ethical controversies (23). Several advantages in comparison to other stem cells are stated here (24). These cells are induced to their neuronal fates by factors such as Edaravone (25) (26) and Resveratrol (27). Differentiation is also known to be induced by multiple growth factors and reagents, but sometimes the immunological response of these factors to the scaffolds cause allodynia[2]. Hence alternatives without chemical disturbance, such as ***electrical stimulation*** have been employed lately to induce cells to specific fates (28). Carbon nanotubes (29) spread uniformly across any scaffold is known to increase the electrical conductivity of the material, thus making electrical stimulation possible.

Importance of the proposed project in the context of current status

Various techniques and methodologies have been tested for Traumatic Brain Injuries in general. However, very little medical treatment (only for secondary damages that occur as a result of DAI, such as hypoxia and seizures) is provided for patients with DAI. Neural tissue engineering approaches are

[2] increased pain to a stimulus that is otherwise noxious

also not widely established for DAI. This project will mainly focus on proposing a solution to DAI in the prefrontal lobe amongst other TBIs while ensuring a minimally invasive technique for implantation of the scaffold and regeneration of the neurons. The novelty of this solution is the combination of the best techniques to ensure better emulation at the site of injury. This project, brings together the best in all the above details, i.e., hUMSCs to be differentiated along with their DFs in 4RepCT covered with carbon nanotubes, which is later seeded onto injectable HA hydrogel and provided to the site of the injury. Necessary controls are maintained and various analyses are performed to characterise and quantify properties.

The materials employed throughout this experiment are all biocompatible and biodegradable and are known to not elicit adverse effects at the site of injury and not cause astrogliosis or secondary cell death. This solution will be one among the initial ideas for treating DAI, and will certainly pave the path for further research in this area. The idea of differentiating cells on SS matrices by electrically stimulating the scaffold, before transplantation to enhance the efficiency of differentiation and regeneration is novel and the injectability of the hydrogel proves as an added advantage to the entire case.

III Work plan - Methodology

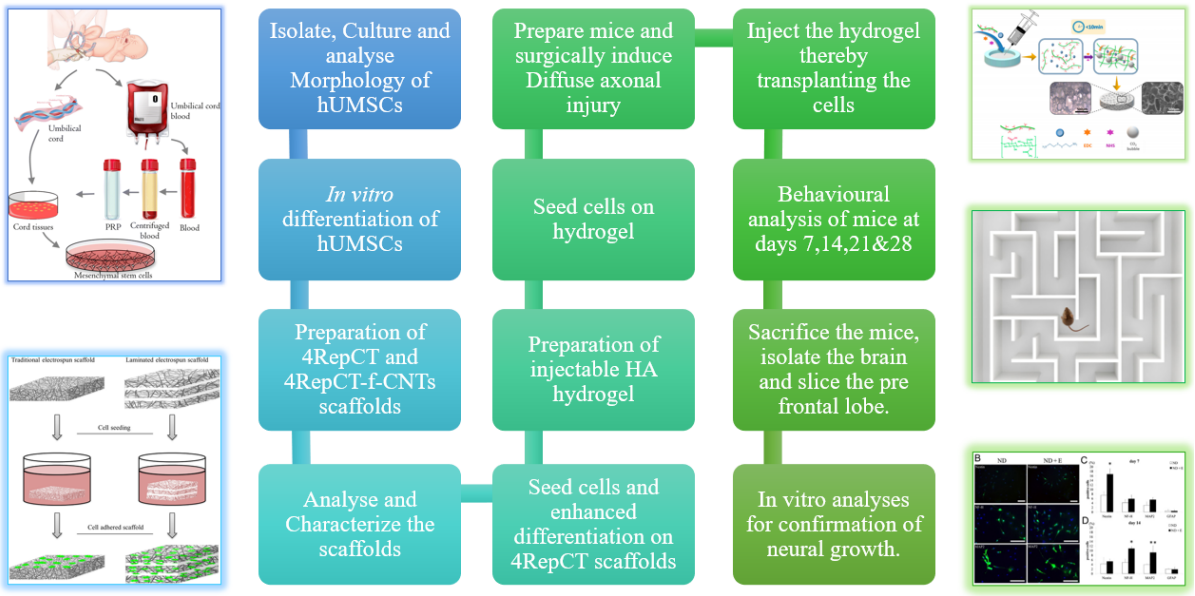

Figure 1 *- Workflow of the Methodology to be followed*

Step 1 - Isolation & Culture of hUMSCs - The hUMSCs are obtained from the umbilical cord. The Umbilical cord is first rinsed thoroughly with D-Hank's medium and then transferred to H-DMEM/F12 culture medium under aseptic conditions, stored at 4^{o}C (26). The blood vessels, which includes umbilical artery, umbilical vein are removed and the mesenchymal tissue is cut into small pieces of size ~ 1mm, which is then treated with 0.2% collagenase II. To obtain the primary cells, digest the tissue in a saturated solution containing EGF, FBS, penicillin-streptomycin mixture as mentioned in (27). The culture medium is replenished every day and this is repeated for 3 days to achieve ~90% confluency and this results in single cells for passaging (30) at the ratio 1:3.

Step 2 - Analysis of the cell morphology - Before proceeding with further experiments and analyses, we'll need to make sure that the isolated cells indeed are the hUMSCs. Several markers have been established for hUMSCs and the most prominent ones are CD11-PE, CD45-PE, CD73-PE, CD90-PE, CD105-PE, HLA-DR-PE, CD19-FITC, and CD34-FITC. Take a part of the isolated cells for this step. Digest the cells with trypsin, rinse it with PBS and aliquot them into 8 tubes. Mouse anti-human antibodies against each of these markers are added into these tubes and they are incubated for 30 mins, after thorough mixing of the contents. The cells are rinsed with PBS again and centrifuges, before analysing them on a flow cytometer (26). Pluripotency can be analysed by immunocytochemical studies which should confirm that colonies of HUCMSCs would express SOX‑10, TRA‑1‑81, and intracellular transcription factors OCT4 and NANOG (31).

Step 3 - Differentiation *in vitro* of hUMSCs to neuronal cells - After 3rd passaging of cells (based on their logarithmic growth phase), split the hUMSCs to two groups - one for further differentiation on 4RepCT matrices and the other for testing differentiation in 4RepCT matrices induced by electrical stimulation. The first group is further divided into four different sub-groups and label them as CTL (control - no DFs added), LD (low levels of DF), MD (medium levels of DF) and HD (high levels of DF). Neurobasal medium containing 50 ng/ml bFGF was first induced for 24 hours. Wash the cells with PBS and add DF: Edaravone + Resveratrol in Neurobasal solution, at concentrations as specified in Table 1. At regular intervals of 6 hours after induction, perform the following *in vitro* analyses - RT-PCR

with appropriate primers and Western Blot with appropriate antibodies for mRNA and protein expression of GFAP, NSE and Nestin (26) (32). As reported by literature, post 12 hours of induction with an optimal concentration of DF (MD and HD), the stem cells begin differentiating into neuron-like cells.

Differentiating factors	**Control**	**Different concentrations of the Differentiating factors (DF)**		
Groups	**CTL**	**LD (low)**	**MD (medium)***	**HD (high)***
Edaravone	0.00 mg/mL	0.65 mg/mL	1.31 mg/mL	2.62 mg/mL
Resveratrol	0.0 mg/L	7.5 mg/L	15.0 mg/L	30.0 mg/L

Table 1 - *In vitro differentiation - groups and concentrations of DFs*

Step 4 - Preparation of 4RepCT scaffold - The recombinant spider silk protein 4RepCT is first produced using *Escherichia coli* as mentioned here (33). The protein solution is purified and sterilized and concentrated to 3 mg/mL by ultrafiltration (Amicon Ultra, Millipore). The protein solution is left overnight to self-assemble to form fibres, which is then autoclaved for sterilization, without affecting its stability and mechanical/ chemical properties. When the dry 4RepCT fibre is mixed with amine-functionalised multiwall Carbon Nanotube (f-CNTs), by applying water drops, followed by pressing and shearing against two Teflon sheets, the fibres turn black, contract in size and is uniformly covered with f-CNTs which provides the 4RepCT, the ability to be electrically conductive. Further details of the procedure are explained in (29). The fibres in the dried matrix intertwine in a non-woven fashion creating a 3D miniature environment with pore-like cavities (~ 5 - 30 µm)

Step 5 - Analysis of the scaffold - ***Chemical characterization*** **-** Fourier Transform Infrared Spectroscopy and Raman Spectroscopy are performed before and after adding f-CNTs to the 4RepCT fibre. An expected result is that there are no additional disorders that show up as a consequence of coating the matrix with f-CNTs. Cell adhesion is naturally facilitated by 4RepCT and hence, addition of supporting peptides is not essential. The adhesion properties can be characterized by contact times of cells with the scaffold as explained here (34). ***Mechanical characterization-*** The stress-strain curve (performed using Dynamical Mechanical Analyser) is constructed by applying weights that are changed

frequently and the Young's modulus is calculated from the slope of the initial linear portion (35). This test would suggest that the tensile property of the new material should be less than that of the original one. The procedure for resistivity measure is elaborated in (29). ***Physical characterization-*** SEM and TEM image analysis will help us study the porosity of the scaffolds and characterize the surface morphology. This is done by coating a thin layer of gold over the surface and analysing (35).

Step 6 - Enhanced differentiation using 4RepCT matrixes - As mentioned earlier, there would be two groups - 4RepCT fibres in a matrix and 4RepCT coated with f-CNTs for electrical stimulation. Since differentiation using electrical stimulation has not been tested earlier for hUMSCs, the methodology is designed to test the same and compare the levels of both the groups.

Seed the cells (obtained after Step3 - the CTL, MD and HD groups) onto the matrices of both the groups. For the second group, pass small electric currents with a varying amplitude as mentioned in Table 2, at 10 Hz, 2.0 ms for 30 minutes using a simulator (SEN-2201, Nihon Kohden) through the matrix on days 0, 1 and 2 after seeding (28) (36). Variability can be introduced by changing the stimulation conditions (10-100 Hz, 10 mV - 50 V, 1 ms - 5 ms, 10 mins -50 mins). This will provide us with 3*3 = 9 groups to analyse.

Types of Scaffolds	Control	Medium concentration of DFs	High concentration of DFs	Amount of electricity
1: 4RepCT only	CTL*	MD (DF)	HD (DF)*	0 mV
2: 4RepCT coated with f-CNTs	CTL (no DF) + LE	MD (DF) + LE	HD (DF) + LE	50 mV
	CTL (no DF) + ME	MD (DF) + ME	HD (DF) + ME*	100 mV
	CTL (no DF) + HE*	MD (DF) + HE*	HD (DF) + HE*	150 mV

Table 2 - *Groups for in vitro differentiation using 4RepCT scaffolds*

Post 7 days after differentiation, the cells are detached from the scaffold and *in vitro* analyses such as RT-PCR and Western blot are performed. It is expected that groups that receive DFs along with electric stimulation would show significant improvement in the expression of neuronal proteins. Based on the results, the groups are considered for the next set of experiments.

Step 7 - Preparation of injectable hydrogel - Hyaluronic acid (HA) hydrogels are prepared with cystamine dihydrochloride as the cross-linker and EDC and NHS as the catalysts. The exact reaction mixtures and stoichiometric ratios are specified here (20). The CO2 bubbles that are formed get trapped *in vivo* (due to shorter gelation time) to form a porous microenvironment, making this a suitable candidate for in-situ injectable hydrogels. Assess various properties of the hydrogel (explained in Step-8) before seeing the selected cell-groups from Step 6 onto the hydrogel's surface.

Step 8 - Characterization of the hydrogel - ***Mechanical properties -*** Rheological measurement (37) (using rotational rheometer) monitors the gelation time by observing changes in the storage modulus (G') and loss modulus (G''). As cross-linking commences, G' increases and literature suggests that 10% w/w hydrogel will take only ~2.5 minutes for gelation *in vivo*. For measuring the swelling ratio, the hydrogel is soaked in PBS and incubated for ~21 days in a shaking motion and it is expected that the ratio is around 2.3. For measuring the *in vitro* degradation rate, the hydrogel is soaked inside Tris-HCl under similar conditions and the expected rate for 10% w/w gel is ~20% (18) (20) (38). ***Physical properties -*** The micro morphologies measured by inverted fluorescence microscope and cryo-SEM should indicate enough space for the cells to form connections and grow, with a porous size of ~450 μm. ***Chemical properties -*** HA is naturally a part of ECM and it promotes cell adhesion by binding to the CD44 receptor on the cell surface. To determine cell proliferation on the hydrogel, contents were determined using Alamar blue over a week, and it is expected that the cell count increases over the week. To see if the hydrogel alters the pH of the surroundings, it was soaked inside PBS and the pH was measured regularly over a week. It is expected that the pH should be quite stable across the days.

Step 9 - Preparation of mice with Diffuse Axonal Injury - Five-Seven week old mice are used for the transplantation. Once they are anaesthetized and fixed on the table, a small skin incision is made and using a metal probe, a minute disturbance (more like a touch) is provided for 30 sec with 30 sec-break three times, in the prefrontal cortex to cause DAI (28). The incision is sutured and the mice are allowed to rest for seven days. Post the resting period, the mice are separated into 6 groups: (i) Control from 4ReptCT Matrix (n=5), (ii) Control from 4RepCT-f-CNTs Matrix (n=5), (iii) and (iv) would be the

groups that performed better in Step 6. By performing a minimally invasive surgery, the HA hydrogels containing the cells (3.0×10^5 cells/hydrogel) are injected inside each mice's brain at the site of injury. Following the transplantation, the mice would be assessed for behavioural and motor skills on day 7,14,21 and 28 after transplantation. The mice would be sacrificed, and their brain will be harvested for further analyses, 28 days after transplantation.

	Control		**Test samples that are injected**			
Groups	CTL	CTL + HE	HD (DF)	HD (DF) + ME*	MD (DF) + HE*	MD (DF) + HE*
Scaffolds	4RepCT	4RepCT-f-CNTs	4RepCT	4RepCT-f-CNTs	4RepCT-f-CNTs	4RepCT-f-CNTs

Table 3 - *In vivo transplanted groups (n=5 for each group) for cells regeneration*

Step 10 - Behavioural Analyses - Since the prefrontal cortex is responsible for the behaviour of the organism and in decision making, it is important to analyse if there is a significant improvement in the behaviour of the mice compared to the control groups. Different kinds of mazes can be designed to study various behaviours and interacting manners (39). However, in this study, we refrain from constructing one wide square box (38 × 38 × 40 cm) and elevated plus-maze (a plus-shaped maze with two open and two closed arms (25 cm long × 7 cm width × 24.5 cm high walls of closed arms). In the first scenario, an 'open field test' is conducted where we analyse the amount of time a mouse spends at the centre vs the periphery of the box. This also includes freezing time, where the mouse refrains from movement. In the second scenario, the number of times a mouse preferred exploring the open arms ('head dips' - referring to the mouse checking out the environment) vs the number of times the mouse explored the closed arms would be noted. A broad expectation is that the mice that received transplanted hUMSCs explored peripheries and the surrounding environment (open arms in the elevated plus maze) much higher than the control groups. We would also observe that mice preferred checking out a new arm rather than visiting an already visited arm. Several other tests such as the Spatial Learning test (for assessing decision making), Anxiety-related behavioural tests, Emotional behaviour test and Y maze exploratory test, can also be performed as described here (39) and here (40).

Step 11 - Analyses of the brain - Following 14 days and 28 days post-transplantation and after conducting the behavioural tests, 3 mice from each group will be sacrificed and their brains are isolated. Immediately after isolation, the brains are frozen with the Tissue-Tek O.C.T™ compound (Sakura Finetechnical) and later sliced into 7-µm sections using a cryostat. These sections are used for neural marker staining with immunocytochemistry (NSE, Nesting, GFAP, MAP-2, etc.). Cell survival and ratios of neural marker proteins would be calculated from five equally spaced sections in the lesion area. It is expected that the cells express these neural-specific markers significantly highly in the group that received DFs and electrical stimulation, and no expression whatsoever in groups (i) and (ii). It will also be observed that there is a higher expression of these proteins on day 28 than day 14, suggesting that the transplantation has aided in neural regeneration.

IV Time schedule for the work plan

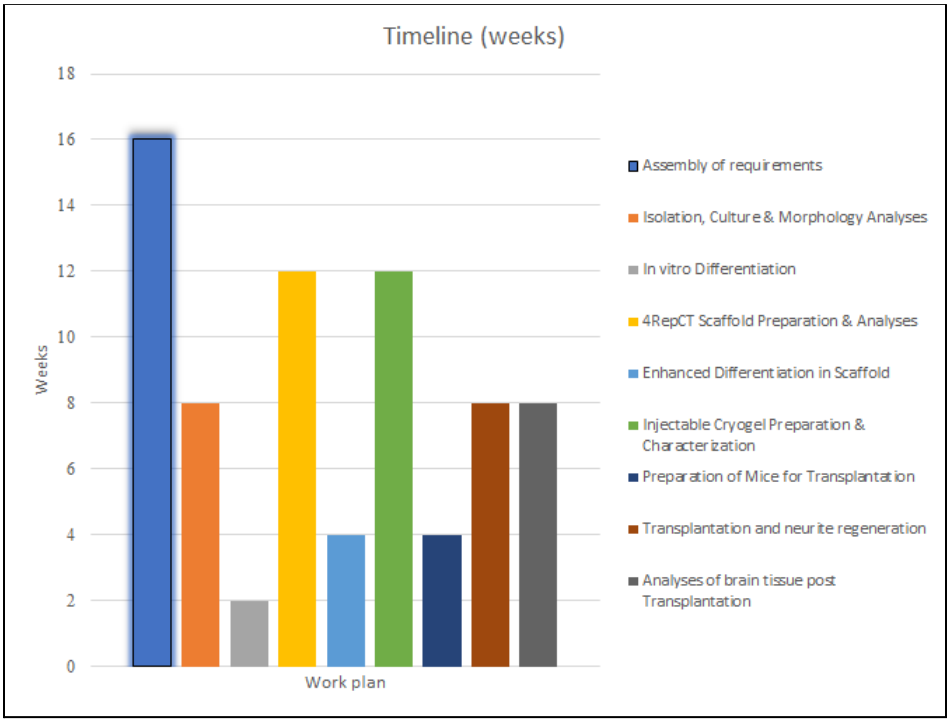

Figure 2 - *Time schedule for the work plan*

Once the funding arrangements are taken care of, the time schedule would resemble the above chart and the whole project (if each step proceeds as planned), would require at least 9 months for completion.

V Plan of action for utilization of the research outcome from this project

The proposed solution, once verified, can be **scaled up** to testing in other higher-order mammals such as pigs and macaques, provided we take care of all the ethical concerns. While scaling up, the procedure for incision and the concentration levels of differentiation factors needs to be modified appropriately so that a significant effect is observed in the case of these mammals. We can further test to see if the 4RepCT matrix can be embedded along with hydrogel inside the brain. The degradation rate and the rheological properties need to be modified accordingly. The concentration and weight % should also be altered to modify the physical/chemical and mechanical properties appropriately. If that is achievable, commercialization of the scaffold can be looked into.

VI Environmental Impact and Risk Analysis

Risks and associated failure are always a part of every planned experimental procedure. Likewise, in this experiment, the following are the potential impacts and risks. Disposal of used chemical reagents and engineered cells need to be carefully monitored and done as per institutional safety regulations, to reduce environmental impact and associated risks. Chemical hazards in the laboratory include all the biochemical reagents and they can arise due to inhalation, ingestion or contact near the eyes. Most of the materials that are used in scaffolds are biodegradable, hence they don't pose a severe threat, but safe handling is always recommended. Secondly, since the latter part of the methodology requires mice for transplantation, safety procedures should be followed in taking care of them and while injecting them with our scaffolds. Ethical issues are always a concern and all the experiments need to be authorized before performing.

VII References

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