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Therapeutic Cloning in the Treatment of Parkinsons Disease using Mouse Models

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Abstract: ***Therapeutic cloning can treat Parkinson's disease using mouse models. Using embryonic stem cells that are matched genetically, the infected tissue is replaced and the mouse is then treated with dopamine producing neurons, the absence of which causes Parkinson's disease. These addition of artificial neurons are created by the mice's own cells in the lab. Stem cells can be grown into any cell. The dopaminergic neurons are developed form these stem cells. Dopamine is a hormone that is responsible for emotions in our body. Dopaminergic neurons are present in the mid brain and are responsible for the movement of muscles. The mouse's own cell is taken when creating dopaminergic neurons because it was proved to be more effective as different samples do not mimic the original cells. Cloning can prove to be one of the most essential step in treatment of many other diseases Diabetes, Cancer etc if all the particular cell lines are formed.***

Keywords: Cloning, Embryonic SC, Mouse, Dopaminergic neurons

1. Introduction

Parkinson's Disease is a disease which affects the nervous systems. It affects the movement of the body. It creates stiffness in the body and makes it difficult for a human to move. The nerve cells are damaged in the brain, which cause the dopamine level to drop.

Dopamine is a type of neurotransmitter, which our body makes in the brain and the nervous system uses it to deliver messages between nerve cells. Dopamine also plays an important role in controlling our emotions, is responsible for love and pleasure and also helps in planning and thinking.

A Mouse model basically means using a mouse in laboratory to study the human aspects of diseases and physiology. Mice are used because they share the same mammalian features with humans and are also targeted with similar diseases that human face. Many other types of models can be used to study such as the animal models which includes a monkey. Monkeys are the ancestors of human population and prove to provide even similar immune responses. Unfortunately not all laboratories can have the benefit of animal models as gaining the permission is very difficult as well as time consuming. They are also quite costly.

Cloning is a laboratory technique that is used to make exact copies of living things such as genes, cells, tissues etc. Somatic Cell Nuclear Transfer (SCNT) is the most common method of cloning where an embryo is made from a body cell and an egg cell. In this technique, an oocyte is taken and a donor nucleus is transplanted. It also has it's advantages in creating personalised medicine. It is a form of medicine that is specifically designed for a particular human by taking their DNA sample and help us identify the diagnosis, treatment and prevention of a disease. (Hong - GuangXie, 2005).

A successful cloning in treating a disease means exchanging the infected cells with new exactly similar cells in order to perform and function perfectly. The question arises that which cells are to be cloned. At first the dopaminergic cells are made from the mouse's IPC (Induced Pluripotent Cells). IPC are type of pluripotent cells that are obtained from the adult somatic cells. IPC are very much similar to embryonic stem cells, thus are majorly used instead of EC because they are quite readily available. (Lei Ye, CortySwingen and Jianyi Zhang; 2013)

As listed above, PD is caused due to lack of dopaminergic neurons. Thus, here the dopaminergic neurons that are already present in the mice's body is to be taken, and then cloned in the laboratory and further will transplanted in the brain. It takes few months for the transplanted neurons to migrate and grow properly. Studies suggest these artificial neurons also mimic the synaptic impulses. After further observation for at least a month, it is deduced that the mouse shows improvement. (Jennifer Warner, 2003)

2. Materials and Methods

2.1 Producing Stem Cells

The stem cells were produced in laboratory using the following protocol: At first, from the healthy mouse the cells were taken from the tail and nucleus was removed. (SCNT protocol). Under sterile conditions, the cell extracted was grown under controlled culture and further the cell was stimulated to divide. (Masahito Tachibana, Paulo Amato; 2013). In the metaphase II oocyte condition, the cells were treated with caffeine. HVJ - E (hemagglutinating virus of japan) was introduced, which fused the donor cells with MII Oocytes and formed the SCNT oocytes. SCNT oocyte was then treated with Ionomycin 6 - DMAP, which activated the oocytes and got exit from the MII arrest. Maternal totipotency factors were activated with the help of electroporation. Electroporation mimics the action of signalling and induces the release of these factors. The cell was developed into an embryo. After 5 days of blastocyst stage, they were treated with TSA (Trichostatin A) due to which the growth rate increased tremendously. The treatment of TSA created NT - ESC (Nuclear transfer embryonic stem cell) lines under the presence of FBS (fetal bovine serum) which are the basis of deriving ESC. (Don P wolf, Roberto moorey and others; 2017).

Fetal Bovine Serum (FBS) is the only serum that can result in forming the NT - ESC because it contains very less

antibodies, thus does not inhibit any actions and has high versatility. It is also used to culture the ESC and preparation of feeder layers. (James N. Petitte; 2007)

2.2 Deriving Nerve Precursors from IPC (Induced Pluripotent Cells)

ESC were cultured on X - ray MEF (mouse embryo fibroblast) in the growth media (DMEM/12 containing 10ng/ml bFGF and KSR 20%) under well defined chemical conditions. Resulting cells were then treated with neural induction media (DMEM/F12 with B27, N2 and Leukaemia Inhibiting Factor) supplemented with SB and CHIR for 7 days. The differentiation of cells was observed and monitored under flow cytometry and immunocytochemistry. The cells were then given a Real time PCR check. After the analysis, the loss of *Nanog* and *Oct4* expression was observed due to the addition of SB, LIF and CHIR in the previous step. *Sox2* expression was unchanged and *Pax 6* was upregulated after the observation of 5 days. After further observation and performing immunocytochemistry, it helped us understand the expression of *Pax6* was induced faster on day 7. *Pax6* positive cells and *Noggin* (an BMP antagonist) were observed with the help of Real time PCR which proved the enhancement of neural induction as these cells and expression are responsible for the creation of brain cells. Double staining the *Nestin* and *Sox 2* proteins was done. After 5 days, the Sox2 expression was diminished and almost 90% of the population on the 7th day had *Nestin* expressed cells. The cells now were given a FACS (Fluorescence activated cell sorting) treatment which gave rise to CD133 positive cells. Almost 98% of the cells were CD133 at the 9th day. The cells were finally differentiated homogenously and these cells were expanded on Matrigel having the presence of CHIR, LIF and SB. These evidence show that all the proteins that were expressed showed enhanced neural response and the final derived cells were the precursors of nerve cells. (Wenlin Li, Woong Sun; 2011)

These precursors were then inserted in the brain of the mouse which had symptoms of PD. After exact 9 weeks, these PC (precursor cells) were migrated and grew into several subtypes of nerve tissues. These also inducted nerve impulses, which means they were properly inserted without any issue.

2.3 Producing Dopaminergic Neurons from IPC

IPC were cultured and MEF (mouse embryonic feeders) were seeded in inactivated form onto the gelatin coated tissue culture at a density of 2.6 x 104 cells per cm2 in MEF medium (1mM Glutamax, DMEM, 10% FBS, 0.1mM non essential Amino acids, 100 U/ml penicillin and 100µg/ml). After 2 days, IPC media (conditioned MEF – 48 hrs, 20% KSR, 100 U/ml penicillin, 4 ng/ml FGF - 2, 0.1mM non essential AA, 1mM GlutaMax) was taken. PSC (pluripotent stem cells) were seeded into MEF. The colonies were then manually passaged for once after every 8 - 10 days, using a FP (fire polished) glass Pasteur pipette.

Table 1: PCR Amplification Primers

Gene Names	*Primer Location*	*Sequence*	*Size (bp)*
Aldh1a1	Sense Antisense	GCTTATCAGCAGGAGTGTTTACCA CTCTTCCATTTCCAGACATCTTGA	150
*CHAT**	Sense Antisense	ATCGCTGGTACGACAAGTCC ATCAGCTTCCTGCTGCTCTG	151
DBH	Sense Antisense	CCACTGGTGATAGAAGGACGAAA GGCCATCACTGGCGTGTAC	120
Foxa2	Sense Antisense	AGGAGGAAAACGGGAAAGAATATAA AAGTAAGACTTCCCTGCAACAACA	150
GAD67	Sense Antisense	ATTCTTGAAGCCAAACAG TAGCTTTTCCCGTCGTTC	617
GADPH	Sense Antisense	GGAGTCAACGGATTTGGTCGTA GAATTTGCCATGGGTGGAAT	150
GLAST	Sense Antisense	GGCTTACTCATTCACGCAGTCA GGTAGGGTGGCAGAACTTGAAG	140
Lmx1a	Sense Antisense	CAGTTCCAGACTCAGGTAAAAGTG TGAATGCTCGCCTCTGTTGA	150
Msx1	Sense Antisense	CTCCCTGAGTTCACTCTCCG CAGGAGACATGGCCTCTAGC	192
Pitx3	Sense Antisense	GGACTAGGCCCTACACACAGA TCCGCGCACGTTTATTTC	151

The cells were maintained at room temperature/ 5% CO2. Cell propagation was obtained through manual dissection. The cell colonies were transferred every week. Differentiation was started after the embryonic bodies were formed. Neural progenitor cells were developed geltrex coated tissue culture with the presence of NEP basal medium (DMEM/F12, Invitrogen 11320, N2 supplements and 1mg/mL BSA). Rosettes were expanded in the same media (NEP), but with the presence of 20ng/ml bFGF for every 2 days. After about a week, these rossets were uplifted, were seeded into small dishes for 3 weeks. This process is done for further differentiation. Cultures were then rinsed 2 times and brain cells were perfused with 4% cold periodate lysine paraformaldehyde at 30 µm on microtome. The cultures were then stained with anti - nexin and anti - Sox2. Next, the cultures were mounted in PGAR (prolonge gold antifade reagent) and analysed using Olympus IX81 image system. The RNA was isolated from the newly differentiated cells. cDNA synthesis took place with the help of 100ng with total RNA reaction with Invitrogen. Further, RNAse H was added (1µl) to all tubes (each) and were incubated at 37 degree Celsius.0.5 µl

template (cDNA) was taken in 20 µl reaction along with PCR mix. The PCR cycles were first denatured at 97 degrees for 3 min, followed by 72 degrees for 10 min. There were specific parameters for cycling: 94° for 30 sec; 55° for 30 sec; 72° for 30 sec. These were followed for the next 30 cycles. The dopamine levels were measured using HPLC (High pressure liquid chromatography). The cells were centrifuged at 10, 000 rpm for around 10 min. The supernatant was removed. The separation of metabolites and dopamine took place using phase extraction column (25µM EDTA, 100 µl/L tri ethyl amine, 75mM dihydrogen phosphate and 10% acetonitrile). There were various stages of differentiation of cells that were observed. Stg I being the lowest having undifferentiated stem cells and stg V being highest having mDA neuron maturation. Stg IV showed the presence of Nexin and Sox 2. The cells of Stg IV were then expanded in the NEP basal culture in presence of bFGF. The rossets of these cells were expressed after 1 week of observation. The cells were kept to further differentiate till Stg V. In the Stg V, after running RT - PCR, *Lmx1a*; *Msx1; Foxa2; Pitx3* and many others were expressed. These were the lineage markers of mDA. In Stg V, we also detected the metabolite 'DOPAC' which is the neurotransmitter of dopamine. It means, dopamine can be metabolized into this substance. This clearly indicated that the Dopaminergic neurons were produced. The mouse sample having the PD symptoms was anesthetized with the help of sodium pentobarbital. The mice was then kept in kopf instrument. A 26 gauge Hamilton syringe was gradually injected containing 6 - OHDA (sigma; 20 µg/ml in 4µl PBS containing 0.2 mg/ml ascorbate). The lesions were verified after 1 month by Columbus instrument. (automated rotomotor system) After the lesions were verified, 10µL of 1 x 106 with HBSS Stg V cells were implanted on the stratium part of the mid brain at the ipsilateral (same side) site of lesion which was created by 6 - OHDA. The mice was given cyclosporine daily for about 2 weeks. The transplanted cells took 1 month to perform action. After 4 weeks of critical observation, the improvement in the mice was seen. Eventually, the mouse was fully treated at the end of 2nd month. (Jingli Kai, Ming Yang; 2010)

3. Result

Stg I iPC are undifferentiated and were further differentiated using the DA differentiation procedure. It is a five step protocol. Stg I – undifferentiated iPC; Stg II – Embryonic bodies formation; Stg III – Formation of NP's; Stg IV – Expansion of NP; Stg V – Maturation of neuron. Rosett like structures were observed when the embryonic bodies (EB) were replated. Cells were further differentiated into Stg III.

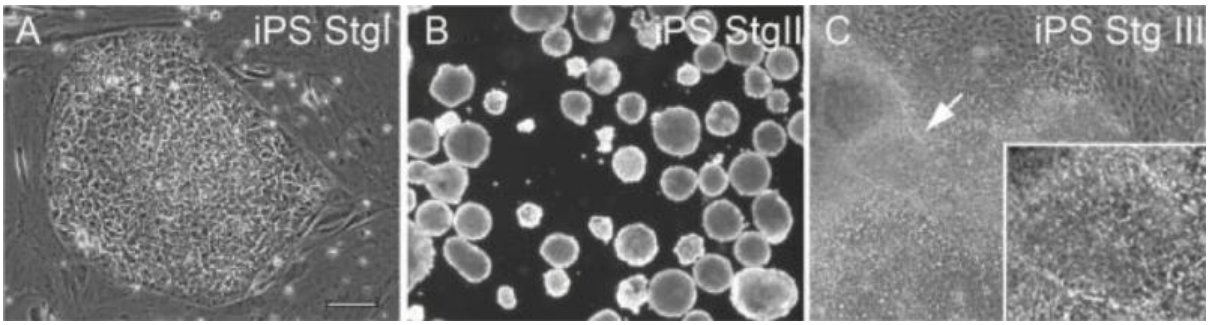

Figure 1: (A) Undifferentiated iPC (induced pluripotent cells) – Stg I; **(B)** Embryonic bodies (Stg II); **(C)** Neural differentiation of iPC (Stg III); Arrow (in image C) depicts the rosettes which is observed during Stg III

At Stg IV, the NP's that are labelled with *Nestin* and *Sox2 (*see Fig.2. (A) and (D) were then further expanded in the basal medium (NEP) along with supplementation of bFGF. Numerous rosettes of Nestin, Sox2 and NP's were formed at Stg IV that expressed specific markers of hNP's called Aldh1a1 and Lmx1a.

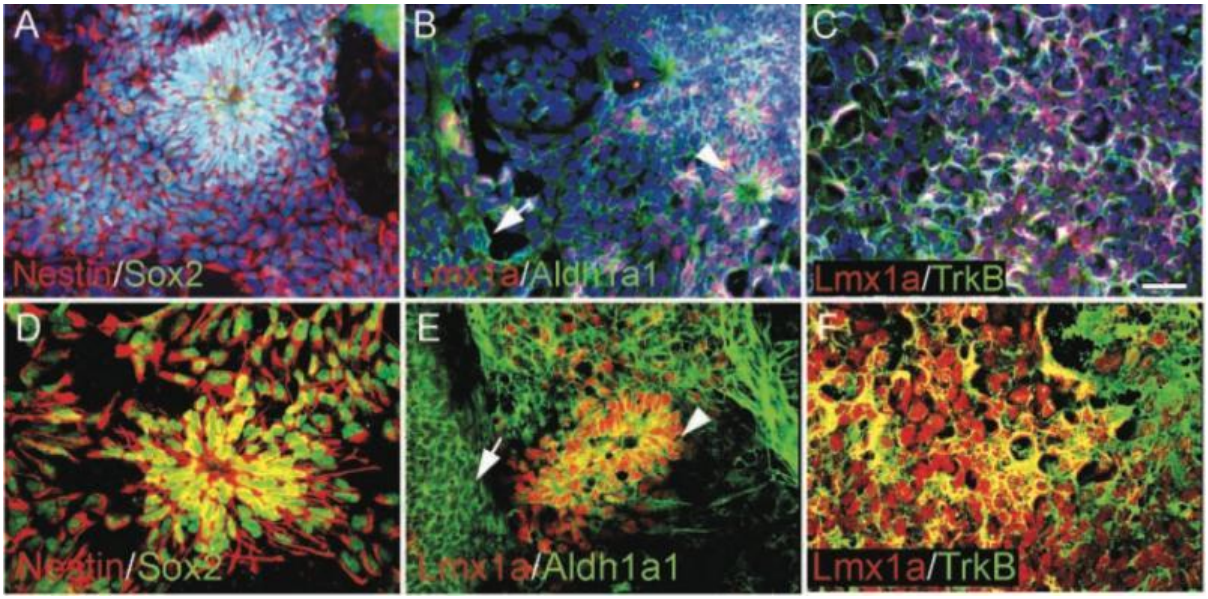

Figure 2: In Stg IV, the expression of the neural markers of iPC. (**A)** – Sox2 (Green) and Nestin (Red) were observed in Stg IV; (**B)** – Dopaminergic neurons in the midbrain and lineage markers were expressed specifically Lmx1a and Aldh1a1; (**C)** – TrkB were expressed on the surface of iPC that were labelled Lmx1a; (**D - F)** – Magnified images of A - C

At Stg IV, harvesting of cells took place and were transplanted. The grafts were observed after 6 weeks, and abundant iPC were found. Furthermore, many differentiated Aldh1a1 precursor cells were observed. These data indicate, that induced pluripotent cells can help in giving rise to dopaminergic neurons in - vivo, but further purification is required in order to eliminate cell heterogeneity.

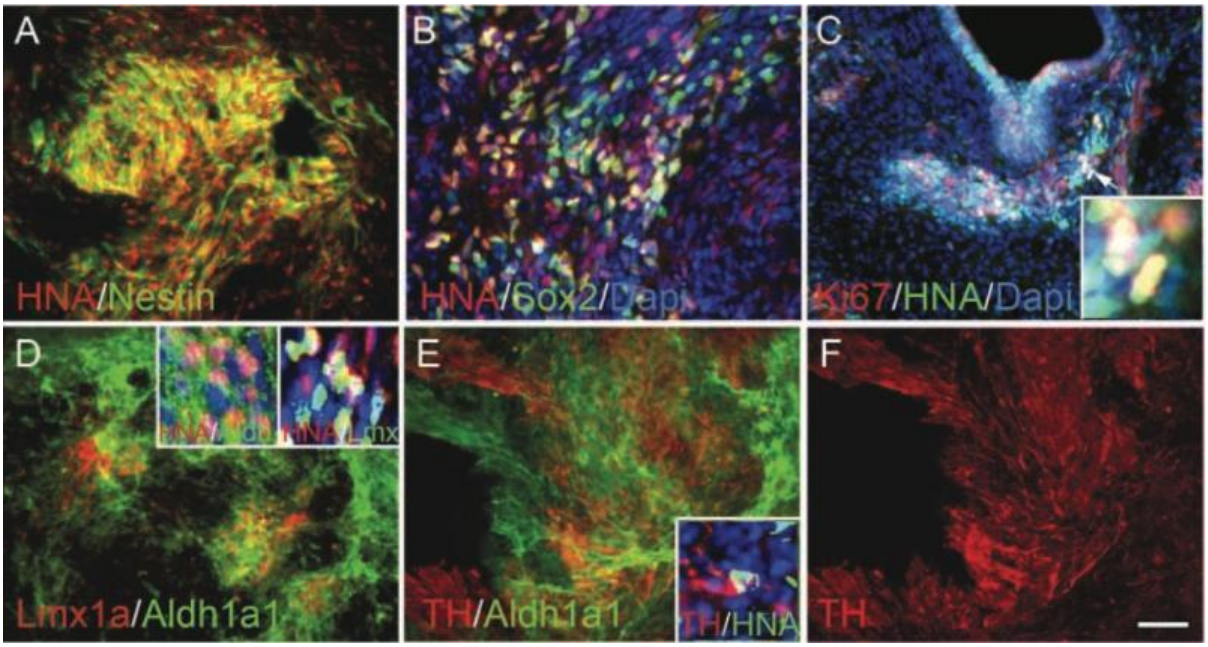

Figure 3: Immunofluorescence analysis of iPC (Stg IV), 6 weeks after the transplantation; (**A)** – Some Sox2 neural cells; (**B)** – Ki67 proliferating cells in grafted cells; (**C)** – Arrowed point shows the magnified image of Aldh1a1/Lmx1a+ precursor cells; (**D)** – precursor cells (NA); (**E and F)** – Mature Aldh1a1 were also detected.

4. Discussion

With a global increase in the aging population, the incidence of Parkinson's disease, a disabling neuro degenerative condition will inevitably present a challenge to an already overburdened healthcare system

The main cause of the present study was to assess the capacity and efficiency of the vectors to infect the midbrain. They still possess two main obstacles and questions regarding treatment in humans, i. e; , how safe would the transplantation procedure might be? and the how long the effect would last.

Even though intense research is needed along with efforts and funding, there are numerous arguments as to why stem - cell based therapy should be promoted and the study for finding the proper solution for vectors and mammalian cell lines for PD patients should continue. The clinical trials with embryonic mesencephalic grafts have provided proof of

principle that DA cell replacement can work and in successful patients can give rise to major, long - lasting recovery of motor function and withdrawal of anti - parkinsonian medication. The human application of stem - cell - derived DA neuroblasts will be based on solid preclinical work in animal models of the disease. It should be emphasized, however, that with respect to efficacy and risk of adverse effects, the requirements for stem cell therapy to become a clinically competitive treatment in PD are high.

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