PURIFICATIONS OF SMALL MOLECULES THAT REGULATE NEURONAL CELL-LINEAGE SPECIFICATION

By

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ABSTRACT OF THE THESIS

Purifications of small molecules that regulate Neuronal cell-lineage specification

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There are certain important factors that are essential for specification, development and maintenance vertebrate nervous system. During development these factors regulate axonal growth, dendrite pruning, cell fate decision and different other neuronal functions.

In adult nervous system, they modulate synaptic activity and regulate neuronal connectivity. Cultivation of mammalian cells for production of recombinant protein has wide scientific and clinical applications because it offers many advantages especially the posttranslational modification. Human embryonic kidney (HEK) 293 cell lines expressing gene of interests will be constructed using calcium phosphate transfection of expression vectors. I have constructed stable cell lines for expressing large quantity of stable neurotrophic and neurological fate determinant factors including: Brain derived neurotrophic factor (BDNF),Glial derived neurotrophic factor (GDNF), Neurotrophin 3(NT3), Noggin and Sonic the hedgehog (SHH).This recombinant proteins were successfully purified by affinity chromatography and are tested on the conversion of induced pluripotent stem (iPS) cells into specific type of neurons, we found that some of the purified proteins are capable of support neuronal differentiation. These neurons have complex morphology, mature synaptic marker such as synapsin, and exhibit mature membrane properties and synaptic activity. We conclude that mammalian cell produced recombinant factors can be used to regulate human neuronal maturation and cell type specification.

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Chapter 1: Introduction

1.1 Mammalian Central Nervous System (CNS) and Stem Cells

The mammalian central nervous system (CNS) is a heterogeneous, complex, highly organized network, and it consists of a tremendous diversity of neuronal subtypes .Each subtype of neurons is generated in a specific spatial and temporal pattern during development. Efforts have been made to understand how such a complex network arises from relatively uniform population of neuronal progenitors. There are various key signaling pathways responsible for the patterning of the mammalian CNS giving rise to a multitude of cell types that contribute to the overall function of the human brain. Although animal models have been helpful tool to understand disease pathology and several gene functions, they do not adequately recapitulate human neurodevelopment [1].

Human embryonic stem cells (ESC) derived from human embryos provide an accurate in vitro model for studying human neurological tissue differentiation. In vitro differentiation of ESC to different kinds of somatic cells represents a rich source for cells that can be used in transplantation therapy [2]. However there are ethical issues associated with the use of human embryonic cells which can be now circumvented by the emergence of induced pluripotent stem cells (iPS) cells, which are generated from somatic cells. The advantage of iPS cells over the embryonic stem cells (ESCs) is the ability to generate patient-specific stem cell lines and avoid the ethical concerns associated with the destruction of a human embryo in addition to the problem that result from rejection after transplantation.[3,4]. Pluripotent stem cells such as ESCs and iPS cells have emerged as a novel tool to study both the normal neurodevelopment and the pathophysiology of human neurodevelopmental diseases.

Human pluripotent stem cell is important tool for understanding brain development and diseases. Stem cells are characterized by*: first*, their ability to proliferate indefinitely and *second*, their ability to differentiate into any organ or tissue specific cells with specific functions [5].

Stem cells can be classified as three types:

1.1.1 Embryonic Stem Cells (ESCs)

ESCs are derived from the inner cell mass of mammalian blastocysts and disappear after five days of development. These cells have the ability to differentiate into cells of any of the three germ layers [6].

1.1.2 Tissue Specific Stem Cells (Non embryonic or somatic stem cells)

Nonembryonic or somatic stem cells are found in specific tissues of adults, children or fetuses .These cells have the ability to differentiate into cells of their tissue of origin, example the neural stem cells give rise only to neurons and support cells of the brain but it cannot differentiate into other types of cells [6].

1.1.3 Induced Pluripotent Stem Cells (iPS cells)

The iPS cells are cells that are taken from somatic tissues for example the skin or blood either from a child or adult (such as skin or blood cells) and are then genetically reprogrammed to behave as ESCs. They have the ability to form all adult cell types as the name pluripotent implies [6].

In this study I applied the small molecules that I cloned and purified (BDNF, GDNF, NT3, Noggin and SHH-N) and used the purified protein to reprogram iPS cells to neurons, thus here, I will describe in more details about iPS cells.

1.2 iPS cells

iPS cells were first created from mouse cells by Shinya Yamanaka, an investigator with joint appointments at Kyoto University in Japan and the Gladstone Institutes in San Francisco in 2006[3].

They genetically reprogrammed somatic cells to an embryonic stem cell–like state by expressing genes and factors important for maintaining the defining properties of embryonic stem cells. This discovery can leads to the establishment of an unlimited source of any type of human cell needed for research and therapeutic purposes and eliminate the ethical concern associated with using ES cells that arise from human embryos.

This experiment was done based on previous findings which proved that gene expression in differentiated cells remains dynamic and reversible. Earlier experiment showed the possibility of activating silent genes in specific cells by fusing the cells with a different cell type. Other studies found that introduction of specific transcription factors could convert specialized cell types from one type to another.

Shinya Yamanaka and his coworkers [3] created murine ES-like cell lines from mouse embryonic fibroblasts (MEFs) and skin fibroblasts by screening twenty-four genes that were previously shown to be expressed in ES cells, and used retroviruses to deliver these genes to fibroblasts from mice. Only four of these transcription factor genes encoding Oct4, Sox2, Klf4, and c-Myc were necessary and sufficient to obtain ES cell like colonies [3].

A year later, at 2007 Yamanaka [4] has generated human iPS cells from adult human somatic cells using the same four transcription factors. Real time polymerase chain reaction (RT-PCR) and Western blot have revealed that the resulting cells express undifferentiated embryonic stem cells markers such as SOX2, OCT3, 4, and NANOG, and also express hES specific cell surface antigens such as SSEA3, 4., (TRA)-1-60, (TRA)-1-81. Just like ESC the generated iPS cells have the ability to proliferate exponentially and differentiate to other somatic cell types such as neurons and cardiac cells.

The human iPS cells that Yamanaka has generated are different from the murine iPS cells in many aspects but they are similar in pluripotent features [4].

1.2.1 Oct-4 (Octamer-Binding Transcription Factor 4)

Oct-4 is a protein that in humans is encoded by the POU5F1 gene. Oct-4 is a homeodomain transcription factor. This protein is critically involved in the self-renewal of undifferentiated embryonic stem cells.

1.2.2 Myc (c-Myc)

Myc is a regulator gene which is that codes for a transcription factor. The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation.

1.2.3 SOX: SRY (Sex Determining Region Y)-box 2

SRY is a transcription factor that is essential for maintaining self-renewal, or pluripotency, of undifferentiated embryonic stem cells and neural stem cells.

1.2.4 KLF4 (Kruppel-Like Factor 4)

KLF4 is a member of the KLF family of transcription factors and regulates proliferation, differentiation, apoptosis and somatic cell reprogramming.

This combination of transcription factors can be functionally replaced by related transcription factors, miRNAs, small molecules, or even non-related genes such as lineage specification factors. These iPS cell lines exhibit similar morphology and growth properties as ES cells and express ES cell-specific genes.

To confirm iPS cells pluripotency, they transplanted these iPS cells in mice, they found that they have formed germ-cell-tumor (teratoma)-containing tissues from all three germ layers.

Mouse iPS cells were similar to ES cells in morphology, proliferation, gene expression, surface antigens, epigenetic status of pluripotent cell-specific genes, and telomerase activity [3].

1.3 Induction of Functional Neurons from Human Induced Pluripotent Stem Cells and Fibroblast

The iPS cells derived neurons are rich source of human neurons to model neurodevelopmental diseases, drug screening, diagnostics and cell replacement therapy [5].

Many scientists have succeeded in converting human ESCs and iPS cells into functional iN cells in less than 2 weeks by retroviral or lentiviral transduction of specific transcription factors. The induced neurons were used to study different neurological diseases.

In 2008 Wernig and coworkers [7] have successfully induced neuronal precursors from iPS cells. The neural precursors have differentiated into various neuronal subtypes *in vivo*, formed synapses and fired action potential when they have been transplanted into fetal mouse brain.

The dopaminergic subtype of the iN has improved behavior symptoms of rat model of Parkinson disease when they have been grafted into adult rat brain [8].

Similar study was performed by Fabin Han and coworkers at 2015 [9]. The iPS cells were converted to NSC and dopaminergic neurons in vitro and then neural stem cells (NSCs) were grafted into rat model of Parkinson syndrome. The engrafted NSC has efficiently converted to dopaminergic neurons *in vivo* and improved the asymmetrical motor symptoms of Parkinson disease.

In 2009 Kaumbayaram and his group [10] derived motor neurons from both ESCs and iPS cells. The derived neurons (iN) that are generated from iPS cells are less efficient than the neurons that are generated from ESC because of unknown reasons. However in both cases the generated iN are expressing neuronal progenitor markers such as Brn2, Sox3, and Pax6.

In 2010 Zhang N, and his colleagues [11] created human Huntington disease (HD) cell model in order to study the pathogenesis of HD. They established iPS cell line from HD patient and converted them into neural stem cells (NSC).Their next step was to generate iN from the iPS derived NSC.

The iPS derived induced neurons technology was used to study the mechanism underlying Rett syndrome in both mouse model [12] and human model [13].

Induced neurons (iNs) can be made directly by the reprogramming of fibroblast without the need to generate the intermediate pluripotent state. At 2010 Vierbuchen and coworkers [14] have tested nineteen lineage determining transcription factors to reprogram mouse embryonic and postnatal fibroblast into neurons. They found that only the combination of three factors (BAM: Ascl1, Brn2, and Myt1l) were sufficient to

induce neurons from mouse fibroblast. The neurons which were produced by this method were able to form synapses and to fire action potential [14, 15].

At 2011 Pang and his colleagues have screened twenty factors and observed that forced expression of the same three factors (Ascl1, Brn2 and Myt1l) can convert human pluripotent stem cells into functional neurons [16]. In addition to that they could reprogram fetal and postnatal human fibroblast into neurons by the addition of the basic helix-loop-helix transcription factor NeuroD1 to the previous three factors. The generated iN neurons expressed pan neuronal markers and in the same year Caiazzo et al. have converted human fibroblast into specific type of neurons (dopaminergic neurons) by forced expression of three set of transcription factors Ascl1, Nurr1, and Lmx1a [15].

Yoo and co-workers have set a nonconventional method in generating neurons from human fibroblast [17]. The new method is based upon the role of chromatin remodeling complexes in neurogenesis. They found that expression of specific microRNA (miR-9* and miR-124) can induce compositional changes in chromatin remodeling process. When they have co-expressed NeuroD1 transcription factor together with the micro RNA ,the efficiency of iN generation has increased by 50%. The efficiency was obtained when they have added Ascl1 and Myt1l.

Amdasaudhan et al have proved that they can induce neurons (ectoderm) from postnatal and adult human fibroblast (mesoderm) by forced expression of a micro RNA (miR-124) and two transcription factors (MYT1L and BRN2) [18].

Furthermore endodermal cells could be also reprogrammed to generate neurons (ectodermal cells) which were proved by Marrow [19].They have induced neurons from terminally differentiated hepatocyte by forced expression of the three factors (Ascl1, Brn2 and Myt1l).

In Pang 2011 study, they have fast tracked neuronal differentiation from iPS cells using BAM [16]. And in 2013, Thomas Südhof and his group [20] have established a rapid and effective method in generating excitatory neurons from ESC and iPS cells using single transcription factor (Ngn2). The induced neurons by this method were able to form synapses and fire action potential.

In 2014 Wen Z. et al. [21] have induced neurons from iPS cells which are derived

from somatic cells of patients with Schizophrenia. They wanted to study DISC1 mutation and its effect on synaptic function.

In summary, this progress, which one can generate large quantity, mature human neurons in a short time frame, has offered a promising approach to study and treat the pathology of neuronal diseases that affect synaptic transmission and synaptic plasticity [14, 15].

Currently researchers are able to derive motor neurons, excitatory (Glutamatergic), inhibitory (GABAergic), dopaminergic neurons and other neuronal subtypes from different parts of the CNS. Production of different subtypes of induced neurons requires trying different protocols and application of small molecules and proteins to guide the process of neuronal differentiation and specification.

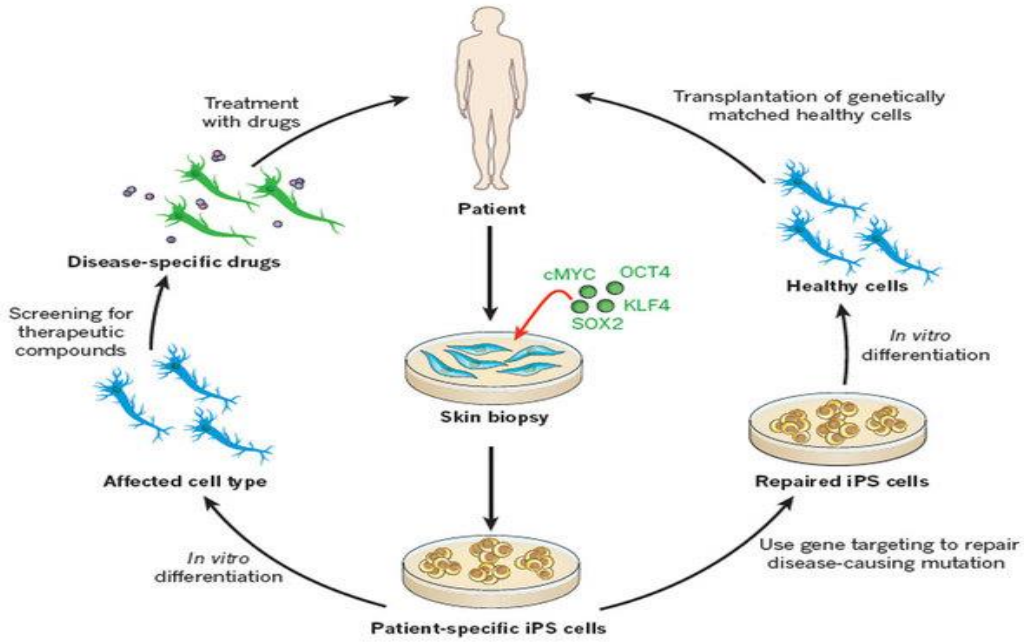


Figure 1: Generation and applications of induced pluripotent stem cells (iPS cells) [22].

A somatic cell source is isolated from the patient. Pluripotency-associated genes are delivered to reprogram the cell. Applications of iPS cells include cell therapy and studies of pathogenesis.

1.4 Methods of Reprogramming Factors Delivery

The production of neurons requires a specific technique for the delivery of reprograming factors. Retroviral vectors have been used initially for the induction of neurons [3, 4, and 23]. Later generations of neurons were produced using lentiviral vector [24, 14] and inducible lentiviruses [25]. The advantage of viral expression vector technique is the self-silencing that eliminates need for timed factor withdrawal [26]. However there are several disadvantages that might affect the efficiency of differentiation. These drawbacks include, (1) permanent genomic integration (2) increased tumor incidence (3) they only infect dividing cells (except lentiviruses which infect both dividing and nondividing cells) [26]. There are critical safety concerns

regarding the integration of viral genome into the iPS cells that are used in regenerative medicine. For these reason scientists has tried nonintegrating approaches to make neurons cells more therapeutically applicable.

Recent studies have used small molecules to induce neurons .These molecules are either activator or repressor for specific pathways that are required for neuronal fate specification [25]. However using small molecules did not yield high induced neuronal survival rate after transplantation combination of viral expression vectors together with application of small molecules has increased the efficiency of neuronal induction and improved their in vivo transplantation success rate [14].

In this study I will use a novel method of using doxycycline inducible lentiviral vector together with purified neurotrophic factors to guide neuronal differentiation from iPS cells. I will also try to test whether purified neurotrophic factors and signaling molecules can accelerate and enhance the differentiation capability of iPS cells compared with commercially available neurotrophic factors.

1.5 Recombinant protein Production

Recombinant proteins can be produced by the integration of recombinant DNA into the genome of living cell such as bacteria, yeast or living cell line such as human kidney cells 293 (HEK293). I used a strategy called epitope tagging to detect and purify the five recombinant proteins. A fusion protein composed of the entire protein of interest plus a short peptide of 8-12 amino acids called epitope which can be recognized by commercially available antibody (Fig.2) [27].

1.5.1 Cloning

Cloning is a man-operated process of extracting DNA (recombinant DNA piece) from a donor cell and implanting it to replicate and function in another type of cell (host cell) .This technique is widely used in biological and medical purposes [28].

1.5.1.1 Flag

Flag is a polypeptide protein tag that can be added to the sequence of any protein during the process of making recombinant protein. Its motif sequence is

DYKXXD. Adding a Flag-tag to this protein allows easy detection of the expressed protein with monoclonal antibody that recognize the flag sequence without the need to design specific antibody for each recombinant protein [29].

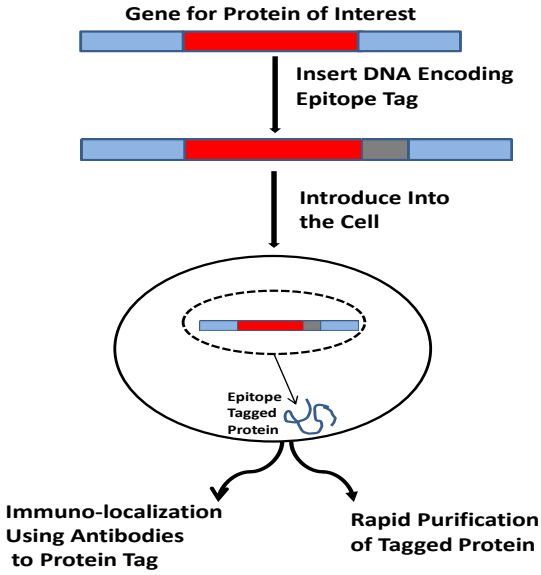


Figure 2: Epitope tagging technique for purification and detection of purified protein [27.]

Short epitope tag (red) can be added to the desired protein (orange) using standard genetic engineering techniques. The resulting construct contains the protein being analyzed plus a short peptide.

1.5.2 Transfection

Transfection of adherent cells HEK293 cells with each of the five proteins vectors (BDNF, GDNF, NT3, Noggin and SHH-N).

HEK 293 is a specific cell line derived from human embryonic kidney cells which are characterized by their ease of growth and transfection making their use is common for transient transfection and production of recombinant proteins or viruses in biological research [30].

Calcium transfection of adherent cells is an efficient method to insert nucleic acid into mammalian cells. The DNA interacts with calcium phosphate to form a crystal that move near to mammalian cells surface and makes their endocytosis easy by the cells [31].

1.5.3 Protein Purification

1.5.3.1 Affinity Chromatography

Affinity Chromatography is a unique technology for protein purification; it is used to purify biological molecules depending on their chemical structure or biological function. It is based on reversible biological interactions between two molecules, such as interactions between enzyme and substrate, receptor and ligand, or antibody and antigen. One of the interacting molecules is called affinity ligand, which is attached to the beads and the other molecule is the mobile target molecules. In this study I used protein A as an affinity ligand that is bound to the agarose beads [32]. Protein A is known for his ability to bind Immunoglobulins which are attached at the C-terminus of the five proteins that I have purified (BDNF, GDNF, NT3, Noggin, and SHH-N).

Chapter2: Neurotropic Factors and Small Molecules

2.1 Neurotrophins

Neurotrophins are group of proteins secreted in the nervous system at low concentrations that promote the development, function and survival of neurons. They are also known as nerve growth factors and they bind to either the p75 and Tyrosine kinase (Trk) family (TrkA, TrkB, TrkC) of receptors. Trk receptors promote neuronal growth and differentiation through the activation of Phosphoinositide phospholipase (PLC), Ras/MAPKmitogen-activated protein kinase (Ras/MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways. These pathways eventually lead to the activation of a transcription factor, CREB (cAMP response element- binding), which in turn act on their target genes. The neurotrophins are first synthesized in target cells and, after that they bind their specific receptor. The next step is their retrograde transport from the nerve terminal to the cell body where they enter the nucleus and regulate gene expression [33, 34].

Because of their effect in neuronal survival and growth, many researches have been done to discover their therapeutic effects in neurodegenerative disorders and physical trauma to the brain and the spinal cord. Neurotrophins play a unique role in the development of excitatory and inhibitory neurons in the developing hippocampus [35].

Neurotrophins play an important role in dendritic growth, synapse formation and synaptic transmission. They play an important role in regulating the balance between cell survival and apoptosis. This group of proteins includes; nerve growth factor (NGF), brain derived neurotrophic factors (BDNF), neurotrophin 3(NT3), neurotrophin 4 (NT4/5) [36].

Recent studies have shown that neurotrophins are involved in activity dependent synaptic plasticity. Neurotrophins synthesis increases in the brain during seizure attack and sensory stimulation [37].

In order to better evaluate the effect of neurotrophins on neuronal differentiation I created stable cell lines for production of this important group of proteins. These proteins include BDNF and NT3 neurotrophic factors in addition to three small signaling molecules GDNF, Sonic Hedgehog (SHH) and Noggin. Many studies have proved the importance in neuronal development and specification *in vivo* and in vitro.

2.2 BDNF (Brain Derived Neurotrophic Factor)

BDNF is one of the most active neurotrophins, The BDNF protein is encoded by the *BDNF* gene, which is located on chromosome 11 13p.It is initially synthesized in the endoplasmic reticulum as a precursor protein, pre-pro-BDNF, which is then converted to pro-BDNF by removal of the signal peptide. Pro-BDNF is then cleaved to generate 14kDa mature BDNF [38]. BDNF binds two receptors on the surface of cells that are capable of responding to this growth factor, Tyrosine kinase B (TrkB) and the low-affinity nerve growth factor receptor (LNGFR also known as p75).

Research has shown that a SNP in the BDNF gene is associated with several diseases such as schizophrenia and alcohol disorders.

In the brain, the BDNF protein has an important role at the synapse, particularly in regulating experience-dependent synaptic plasticity which is essential for learning and long term memory. [39]. BDNF deficiency has been implicated in the pathogenesis of Alzheimer's disease, Huntington's disease, and depression due to its essential role in hippocampal long term memory [40].

BDNF effect on synaptic plasticity is mediated by the activation of TrkB receptors. When BDNF binds to the TrkB receptors, the receptors dimerize and autophosphorylate one of their tyrosine residues, which activate phospholipase C (PLCγ) pathway. The activated PLCγ translocates to the plasma membrane and cleaves phosphatidylinositol 4, 5-bisphosphate (PI (4, 5) P_2) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). DAG stimulates protein kinase C (PKC), while IP_3 releases Ca^{2+} from internal stores by binding to IP_3 receptors. These signaling pathways regulate gene transcription via CREB transcription factor, synaptic proteins translation and trafficking [41].

With the presence of the Val66Met single nucleotide polymorphism in BDNF, hippocampus-dependent learning is compromised due to a reduction in the level of secreted BDNF. The reason behind this is not fully understood but this might be attributed to the misfolding of BDNF protein which in turn leads to inefficient sorting of the pro BDNF protein in the Golgi apparatus [42].

Scientists also found that heterozygous BDNF knock-out mice exhibit a marked reduction in long-term potentiation (LTP) which is reversed when BDNF is overexpressed using an adenovirus mediated technique [43, 44, 45].

The attributed the effect of BDNF in potentiation of LTP due to its role in modulating the synaptic vesicles at the presynaptic nerve end and thus it increase the synaptic strength via increasing the number of docked vesicles at the active zone [46].

BDNF enhances the transmission of excitatory neurons specifically by increasing the release of glutamate at the synaptic end [47]. BDNF has also postsynaptic effect because it increases the phosphorylation of NMDA type glutamate receptor [48].

Several studies have revealed that infusion of BDNF in the ventral tegmental area and the nucleus accumbens potentiates cocaine associated reward behavior [49, 50].

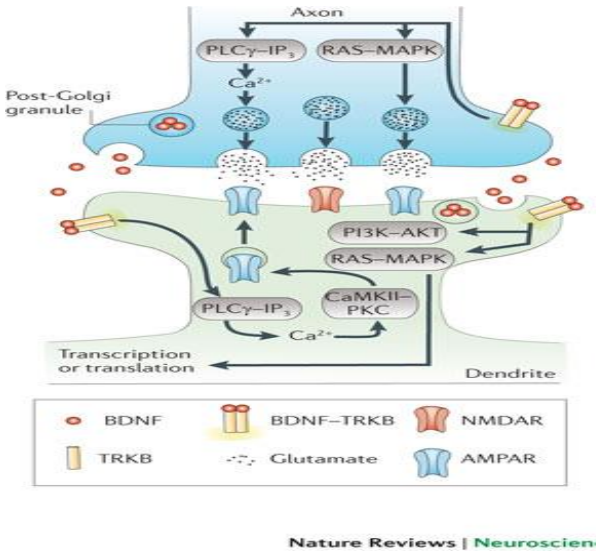


Figure 3: Mechanism of action of Brain derived neurotrophic factor

BDNF binds to–tropomyosin-related kinase B (TRKB) and enhances the presynaptic release of neurotransmitter(Glutamate) through the activation of a RAS–mitogen-activated protein kinase (MAPK) and a and a phospholipase Cγ (PLCγ)–inositol trisphosphate (IP_3)-dependent signaling mechanism; which lead to activation of Ca^{2+}/calmodulin kinase II (CaMKII)–protein kinase C (PKC) pathways via PLCγ–IP_3 signaling. This increases the insertion of postsynaptic Glutamate AMPA receptor [51].

2.3 GDNF (Glia Derived Neurotrophic Factor)

GDNF is a small protein that belongs to transforming growth factor-beta (TGFß) superfamily which all contains 7 cysteine residues in the same relative spacing. It is encoded by GDNF gene which is located on 5p13 locus. It is secreted as (51kDa) 210 amino acids pro GDNF which then proteolytically cleaved into disulfide-linked GDNF homodimer that is composed of two (135) amino acids polypeptide chains .It is highly expressed in the striatum of the brain [52].

GDNF is one of the most potent neurotrophic factors that enhance the survival and differentiation of various types of neurons during development such as midbrain dopaminergic neurons, motor neurons, noradrenergic neurons of the locus coeruleus and Purkinje cells.

Outside the central nervous system GDNF is responsible for the development and survival of the enteric neurons and ureteric branching during kidney development [53]. GDNF protein binds a multicomponent receptor system composed of a ligand specific glycosyl-phosphatidylinositol (GPI)-linked protein (GFRa1) and the transmembrane protein tyrosine kinase Ret [41] which in turn activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3 kinase (PI3K) intracellular pathways. In addition to that GDNF activates proto-oncogene tyrosine kinase (SRC kinase) which mediate neurite outgrowth [52].GDNF increases the expression of calcium binding protein Frequenin, in this way it enhances synaptic transmission [54].

GDNF effect is more prominent on dopaminergic neurons (DA) where GDNF receptor GFRa1 are expressed by dopaminergic neurons that project to the striatum through the nigrostraital pathway [55].Scientists have discovered that GDNF increases the number, size and the length of neurites of primary cultured dopaminergic neurons [56].GDNF also increases the level of Dopaminergic neuron specific transcription factors which is responsible for the expression of several gene such as TH, vesicular monoamine transporter (Vmat2), dopamine transporter (Dat) and aromatic L-aminoacid decarboxylase (Aadc) that is involved in dopamine metabolism [57]. Ablation of Ret in DA neurons leads to progressive loss of nigrostriatal DA neurons.

Theories suggested that GDNF prevents apoptosis in the DA neuron because it up-regulates the anti-apoptotic proteins B cell lymphoma 2 (Bcl-2) and Bcl-X via PI3K signaling [52].

GDNF is also proved to have a neuroprotective and restorative effect against dopaminergic neurons degeneration induced by the neurotoxine (6-OHDA in Parkinson disease mice model. GDNF injection in the striatum of those animals leads to decrease in the nigral dopaminergic neuronal loss [58].

Other scientists have reported functional improvement of parkinsonian features together with increased levels of striatal dopamine in GDNF-treated monkey model of Parkinson disease [59].

Many studies have proved the important role of GDNF in many neurodegenerative disorders such as amyotrophic lateral sclerosis 60] due to its effect in prevention against the degeneration that occurs following axotomy of facial and motor neurons of neonatal mice rats, and birds [61].

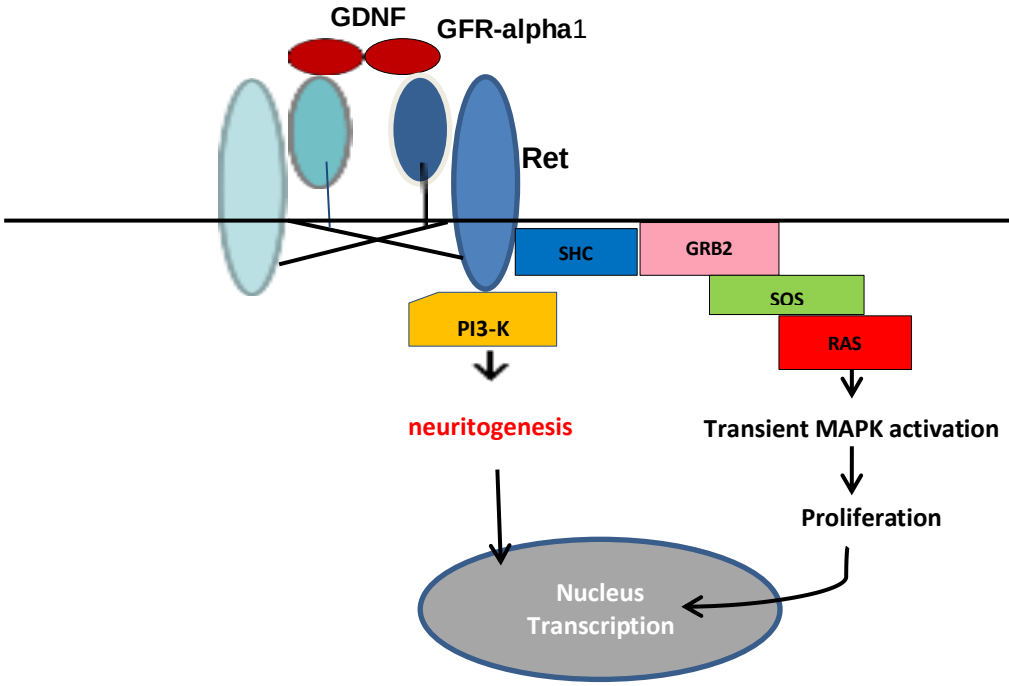


Figure 4: Mechanism of action of Glial derived neurotrophic factor [62].
GDNF glycosyl-phosphatidylinositol (GPI)-linked protein (GFRa1) and the transmembrane protein tyrosine kinase (Ret).This figure represent the TGF-ß/GDNF cooperativity in regulation of neuronal survival.

2.4 NT3 (Neurotrophine-3)

NT3 is a 13.6 kDa protein secreted in low concentration from cells of nervous system and encoded by *NT3 gene* which is located on 12p13 locus . It is the third discovered neurotrophic factor after NGF and BDNF. NT3 structure is closely related to the last two neurotrophins. It promotes its effect via binding to TrkC receptor which is expressed at high levels in neurons of the basal forebrain, the hippocampus, and the neocortex of the mammalian brain [63] .NT3 is involved in the control of neuronal differentiation and maintenance, migration and proliferation. NT3 is synthesized by discrete populations of neurons and astrocytes in the nervous system. NT-3 is also produced and secreted by endothelial cells of brain and choroid plexus capillaries [64].

NT3 is highly expressed in different areas of the brain during fetal development period especially hippocampus, neocortex and cerebellum but its secretion is confined to dentate gyrus in adult life where stimulate the differentiation of neurons derived from NSCs (neural stem cells) of the dentate gyrus neuronal niche. This is how NT3 facilitate learning and memory [64].

NT3 enhance the connectivity of glutamatergic synapse because it increases the glutamate release from the presynaptic membrane and increase of NMDA receptors responsiveness on the postsynaptic membrane [63]. NT3 can also modulate glial cell biology. NT3 is found to modulate myelination in the CNS and promotes oligodendrocyte precursor proliferation, survival, and differentiation. Further, NT3 plays an important role in providing neuroprotection, immunomodulation, and reduces astrogliosis, which has promising significance in treating multiple sclerosis and spinal cord injury [65].

NT3 promotes proliferation and neurite overgrowth in culture neural crest cells and play the same role for in vivo neural crest progenitor differentiation in the dorsal root ganglia [66].

NT3 action is not only confined to the central nervous system, it is also important for the differentiation, development, and maintenance of the proper function of enteric nervous system and sympathetic neurons [67].

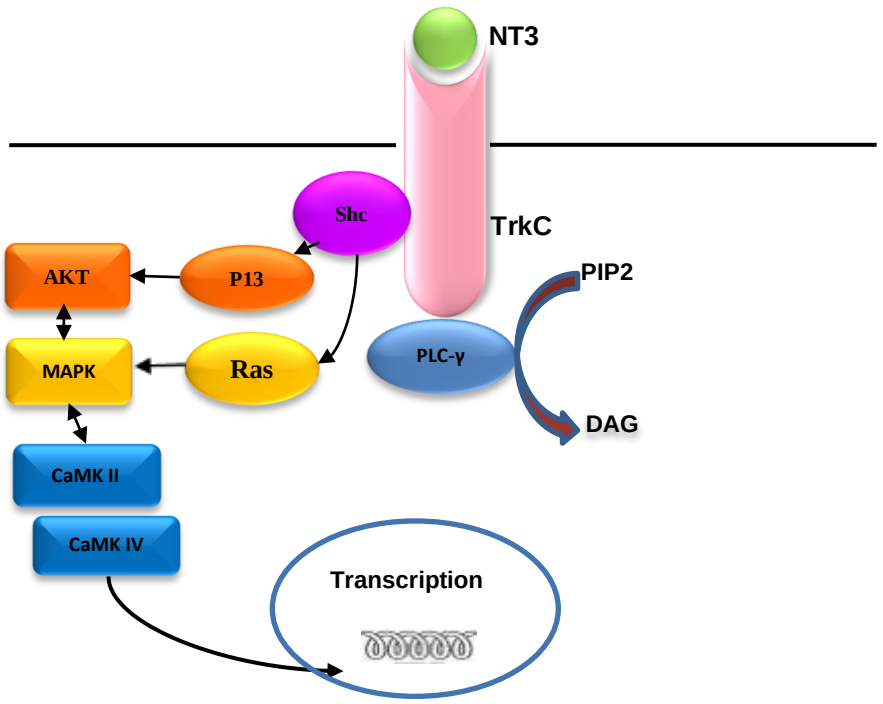


Figure 5: Mechanism of action of NT3.Activation of Tyrosin kinase C receptor (TrkC).

Activation of the T-cell lymphoma invasion and metastasis-inducing protein 1(Tiam1)by NGF/TrkA and NT-3/TrkC requires a physical interaction between Ras-GTP and Tiam1 through its Ras-binding domain. This pathway ends with the activation of protein kinase A which will increase actin remodeling and enhance neuronal neurite outgrowth [68].

2.5 Sonic Hedgehog (SHH)

Sonic the hedgehog a 45kDa protein secreted from the ventral part of notochord (a structure arises from the axial mesoderm) act on maintaining the ventral polarity of the central nervous system and limbs [69]. It plays an important role in formation of the neural plate, ventral surface of the forebrain, the midline between the right and the left side of the brain and the development of the eyes.

The neural plate is a specialized central portion of the neural tube which is the origin of the future vertebrate CNS. SHH induces its effects in a concentration-dependent pattern with high concentration in the ventral part and low concentration dorsally. The active part of the SHH is the 20 kDa N terminal domain which is cleaved from the 25kDa C-terminal domain by a protease within the C terminal domain. SHH molecule is

modified by attachment of lipid molecules represented by a cholesterol molecule which is attached to the C terminal domain and a palmitate molecule which binds the N terminal domain which is important for high potency and efficient signaling of the SHH molecule [70 , 71].

The SHH pathway starts with its binding to the receptor Patched which relieve the repression of Smoothened receptor (a seven transmembrane receptor). In turn this lead to activation of a series of cytoplasmic signaling pathway ends with the activation of zinc finger transcription family Gli. This family includes Gli1, Gli2, and Gli3.Gli1and Gli2 act as transcription activator while the truncated form of Gli3 act transcription repressor. SHH increases the expression of Gli1and Gli2 and inhibits the cleavage of Gli3.The level of SHH will determine the ratio between the activating to the repressor Gli factor (Gli1, 2/Gli3) which in turn decide the dorsoventral fate. The high ratio of activating to repressing Gli specify ventral fate of the neurons because Gli1and Gli2 transcription will activate the expression of ventralizing genes which are Nkx2.2 Foxg 1.Nkx2.2 is vital for the development of two subtypes of GABAergic neurons parvalbumin and somatostatin in the neocortex [72, 102].

SHH later in the embryonic period will be secreted from the medial ganglionic eminence and will play critical role in the specification of the cerebral cortex GABAergic interneurons via the activation of Nkx2.1 transcription factor.

GABAergic neurons release the neurotransmitter GABA which hyperpolarizes the postsynaptic neurons. GABA makes the postsynaptic membrane more negative and by this way it will be hard for the neuron to reach the threshold and fire action potential. By this mechanism they act as a regulator of excitatory neurons and inhibit their hyper excitability

Disorder of inhibitory neurons has been implicated in several neurological illnesses such as Epilepsy, Down syndrome, Schizophrenia, and Autism related disorders (ASD).

Many researches are focusing on using SHH to generate GABAergic neurons from ESCs and iPS cells. By this way the induced inhibitory neurons can be transplanted *in vivo* and replace the function in patients with neurological disorders [73].

Recent studies have shown that the addition of puromorphamine (which is a SHH agonist) to embryonic stem cells culture specify their differentiation into ventral spinal cord progenitors and motor neurons [74].

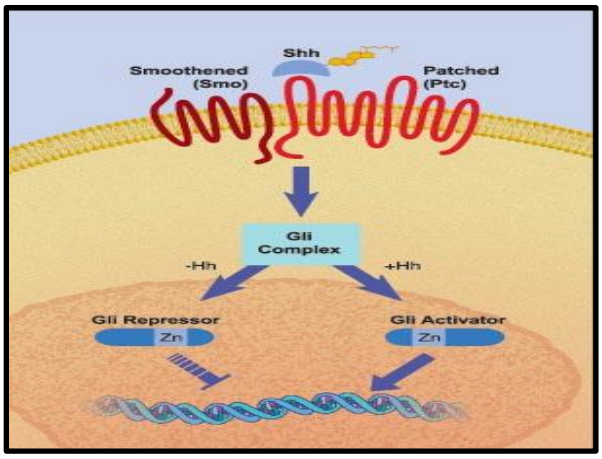


Figure 6: Sonic Hedgehog Secretion and Mechanism of Action [75].

Posttranslational processing of Sonic Hedgehog molecule. After its secretion from the notochord SHH molecule consists of active part (N terminal) and catalytic part (C terminal).
Lipid modification of the molecule enhances its secretion and signaling.

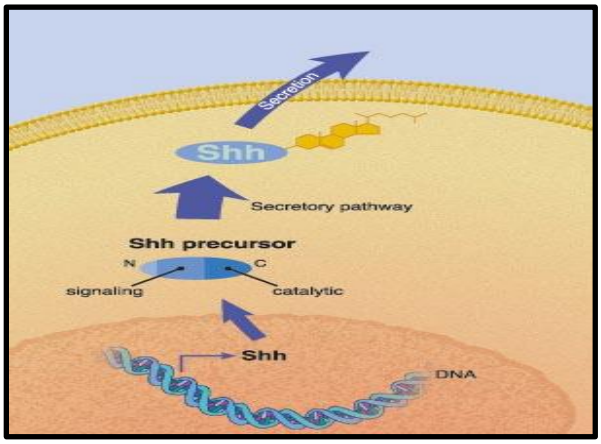


Figure 7: Sonic Hedgehog Secretion and Mechanism of Action [75].

Mechanism of action of Sonic hedgehog SHH-N bind to patched receptor and remove its inhibition off smoothened receptor. Following that smoothened activate Gli activator and inhibit Gli repressor which enhance the effect of transcription factor (CREB) cyclic AMP response element binding protein.

2.6 Noggin

Noggin is a glycosylated homodimer protein released from the dorsal part of the notochord aencoded by *Noggin* gene. It plays an important role in the nervous system, skeleton and somite development.

Noggin functions via antagonizing bone morphogenic proteins (BMP) ligand activity. BMP are group of proteins that belong to transgenic growth factors beta (TGF β) superfamily which are involved in the differentiation and development of different parts of the body such as (bone, muscle, brain, cartilage ,limbs,…etc.) during embryonic period. In the brain BMP is responsible for dorsoventral patterning of the neural tube and somite. To ensure that BMP perform this function properly, it must be regulated by an antagonist represented by noggin [76, 77].

Noggin binds to BMP (BMP2, BMP4 and BM7) at high affinity and thus it inhibits their binding to BMP receptors [78].

During the early embryonic period Sonic Hedgehog (SHH) is responsible for ventral cell fate, but with time this process is lost progressively despite the fact that SHH expression from the notochord is still normal. The secretion of BMP proteins blocks SHH-mediated induction of Pax-1, a sclerotomal marker, whereas addition of Noggin is

sufficient to induce Pax-1. Noggin and SHH both induce Pax-1 synergistically but in different pathway. This explains why inhibition of BMP signaling by Noggin is an essential for normal patterning of the vertebrate neural tube and somite [69, 79].

Noggin also enhances the differentiation of human ESC into oligodendrocyte at specific stage after the addition of retinoic acid (RA) [80].

Noggin enhances the differentiation of dopaminergic neurons from embryonic stem cells [80, 81]. This effect is confirmed by the evidence that noggin mRNA are expressed more prominently in substantia nigra which is rich in dopaminergic neurons [82].

This effect has promising benefit in the treatment of Parkinson's Disease which is a neurological disorder that characterized by the progressive loss of dopaminergic neurons in the midbrain substantia nigra [83]. Dopaminergic neurons are involved in voluntary movements and variable behavioral processes in the brain such as mood, stress, reward and addiction [84].

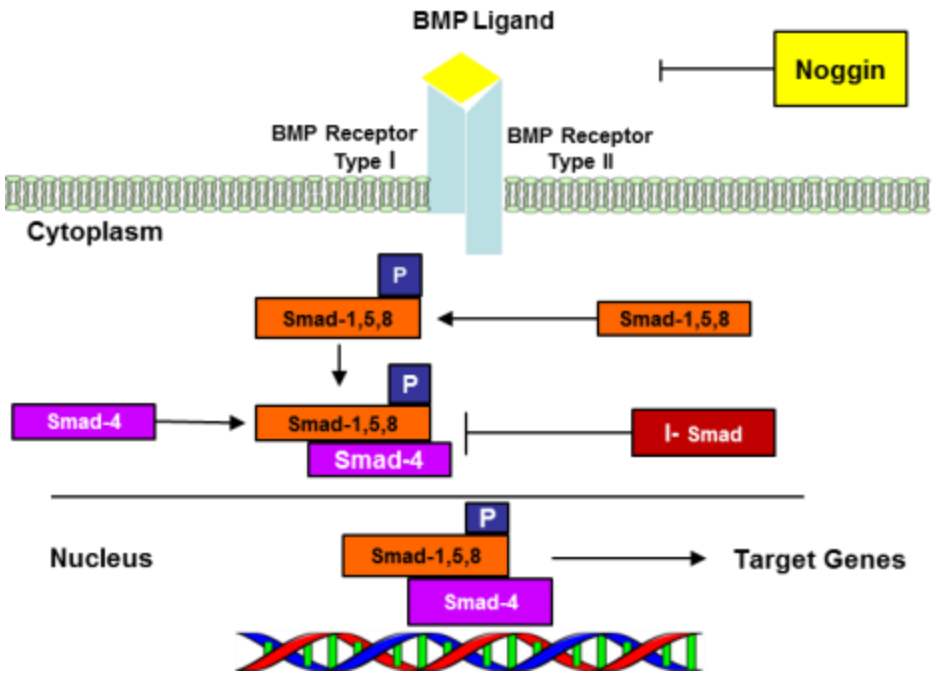


Figure 8: Mechanism of action of Noggin in the central nervous system [85].

Noggin inhibits BMP (bone morphogenic proteins) which in turn inactivate SMAD pathway and decrease expression of target genes.

2.7 Overall Aim and strategy

The aim of my experiment is to create a stable cell line for the production of five proteins and test their function by the application of the recombinant proteins during the differentiation of iPS cells to specific neuronal type.

*Stable cell line: is a way to incorporate the gene of interest into the DNA of the cell to allow its expression for long term. The foreign gene will be part of the cell genome and will be continuously replicated. It is commonly used for the purpose of antibodies and recombinant proteins production.

This goal is achieved by stable transfection of the cell with the gene of interest and co-transfect the cell with selectable marker to select for the cell that has the gene of interest and in the same time select for the cell which is stably transfected from the transient transfection.

Application of the recombinant proteins to test their effect on differentiation and specification of certain type of neurons during their induction from undifferentiated iPS cells by infection with fate determining transcription factors using doxycycline inducible lentiviral vectors.

In this study I applied BDNF, GDNF, and NT3 in induction of excitatory neurons, noggin in induction of dopaminergic neurons and SHH-N in induction of inhibitory neurons to test the effect of these recombinant proteins on cell fate determination.

Chapter 3: Experimental Procedure

3.1 Constructions of Vectors for Expressing Neurotropic Factors

The active CDNA sequence of the 5 proteins (BDNF, GDNF, NT3,Noggin and SHH-N) have been cloned into modified pCMV6-XL4 vector which attaches a flag tag at the N terminus of the protein along with a Immunoglobulin (FC) sequence to the C terminus of each of the five proteins to allow their secretion by the transfected cells have Immunoglobulin (FC) sequence attached to the C terminus of each of the five proteins to enhance the secretion of these proteins by the transfected cells and flag tag.

a. The bacteria carrying cDNA of each of the five proteins were obtained from Addgene.
b. Bacterial culture was done into agar plates with the addition of the respective drug resistance.
c. Well defined colonies were picked and amplified in 8 mL LB, the respective antibiotic were added and incubated for 16 hours.
d. The DNA were exracted from the bacterial culture.(Qiagen miniprep kit)
e. The cDNA region was amplified using primers flanked by XbaI and NotI restriction enzyme cutting sites.
f. The pCMV6-XL4 vector and the PCR product were digested with XbaI and NotI.
g. The pCMV6-XL4 backbone was ligated with the PCR product and transformed into bacteria.
h. The well-defined colonies were picked, amplified and their DNA was extracted by Qiagen miniperp kit.
i. The result ligation products were sent for sequencing to verify the correct sequence of the insert within the vector.

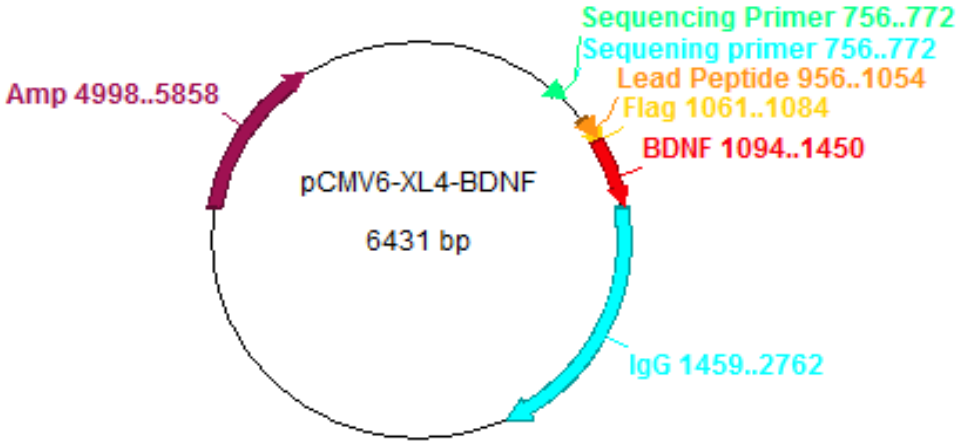


Figure 9: BDNF Cloning Vector

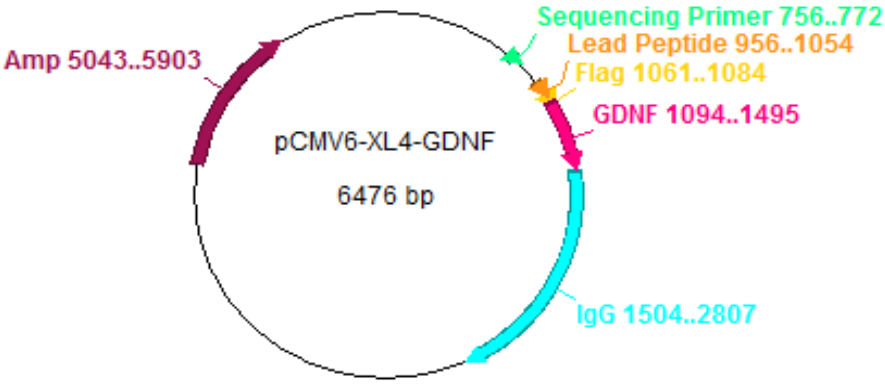


Figure 10: GDNF Cloning Vector

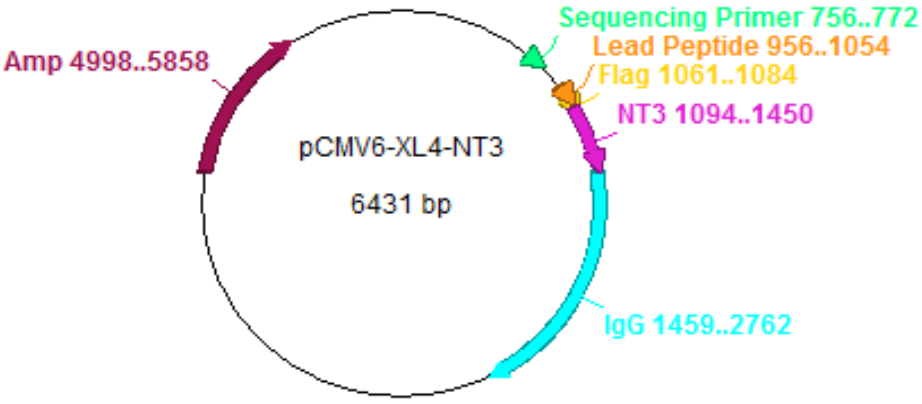


Figure 11:NT3 Cloning Vector.

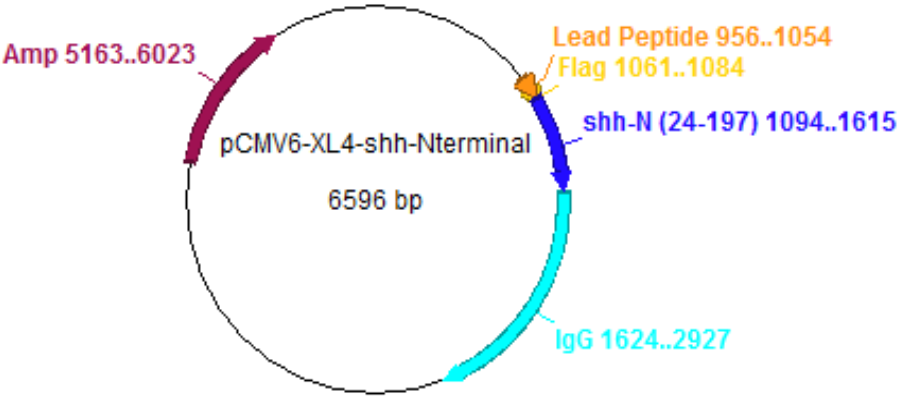


Figure 12: SHH-N Cloning Vector.

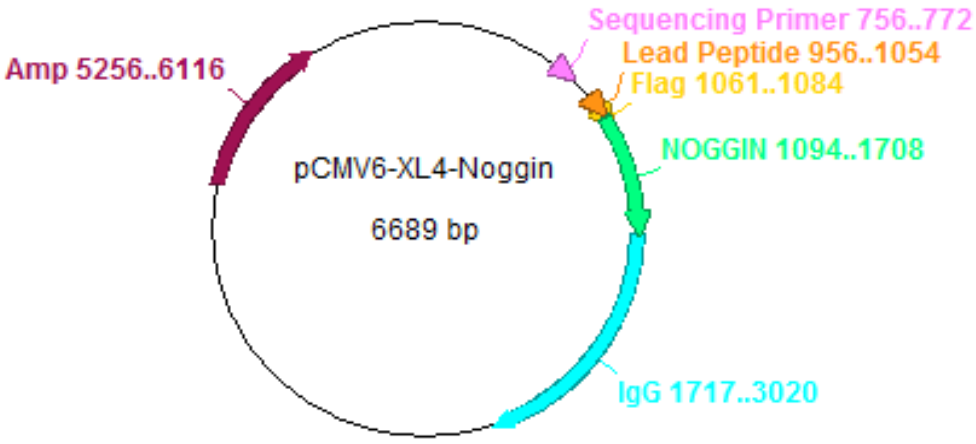
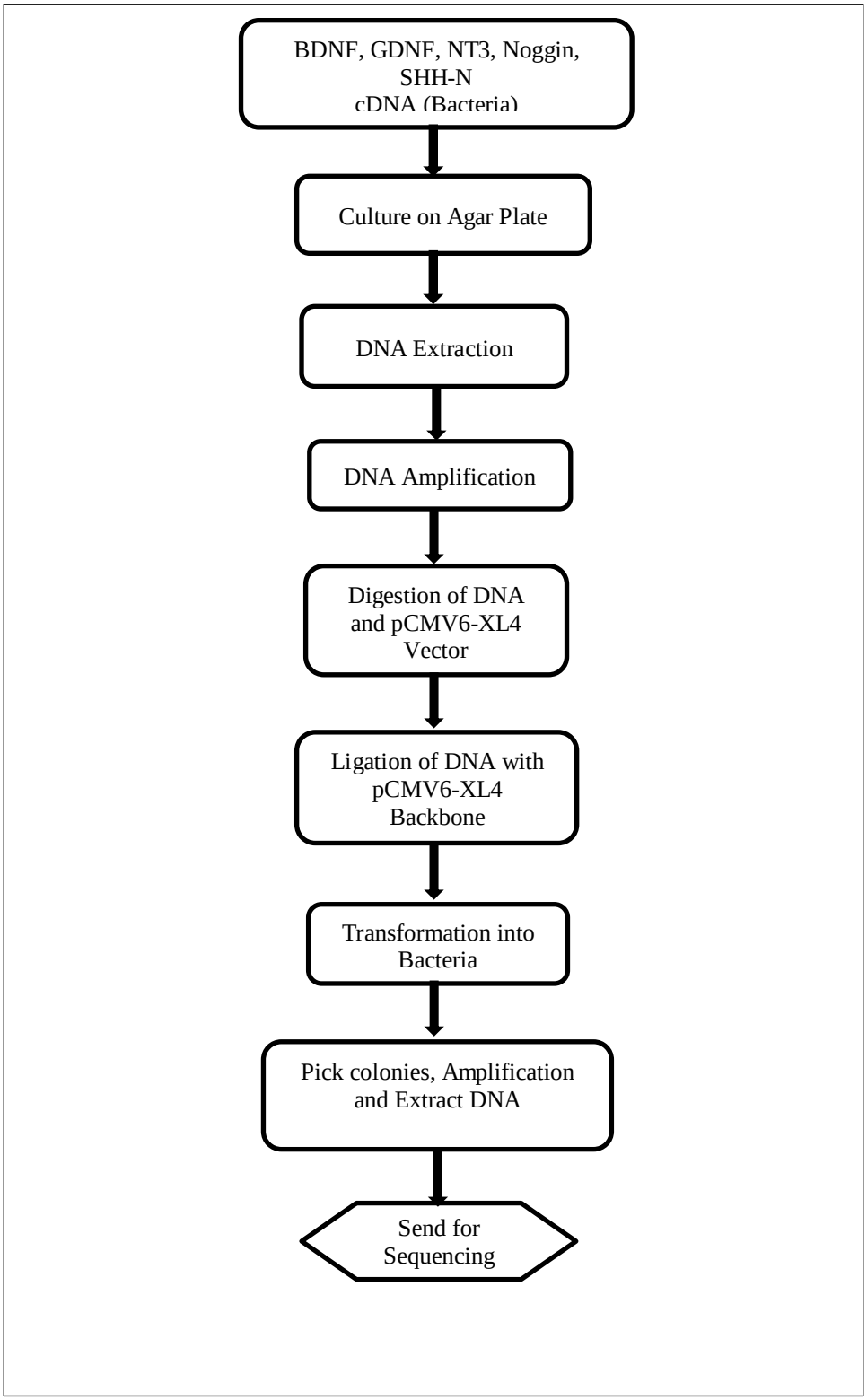


Figure 13: Noggin Cloning Vector.

Flow Chart 1:
Molecular Cloning



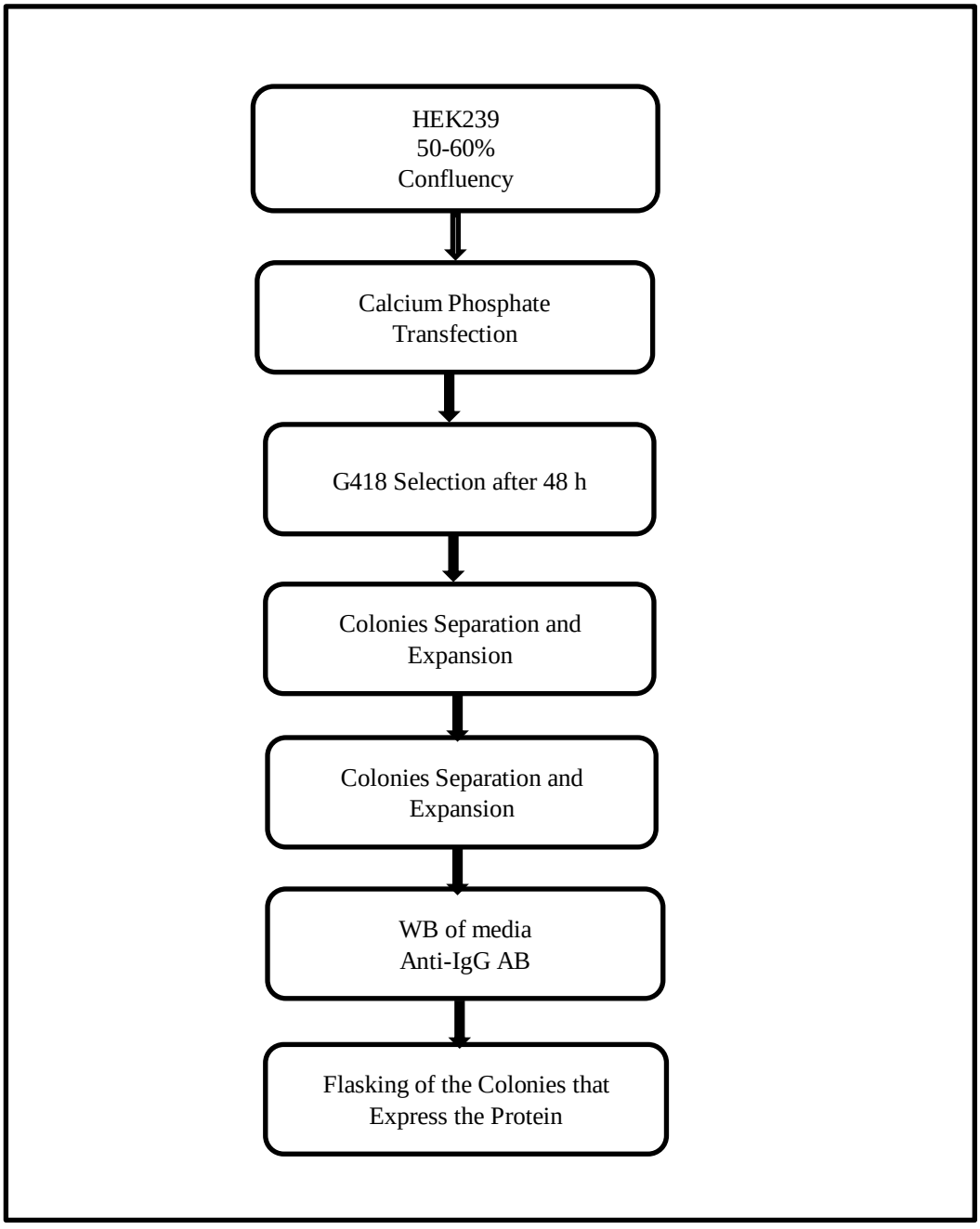
3.2 Transfection

1. Calculation was done to obtain the volume of (9ug) plasmid of DNA and (3ug) of antibiotic resistance plasmid that should be transfected to 100cm plate of 50-60% confluent HEK293 cells.
2. The HEK293 cells were maintained at 37°C and 10% CO_2 in DMEM media that contains (10% FBS and 1% penicillin/streptomycin).The medium was changed one hour before the transfection.
3. Each plasmid was added to the corresponding tube followed by addition of water and 60ul of 2.5 calcium chloride solution (to make 500ul total volumes).
4. The contents were mixed and incubated for 10 minutes at room temperature.
5. The mixture of each plasmid was added drop by drop slowly to a tube that contains 500ul 1X HBS, and the final mixture was vortexed for 30 seconds and incubated in dark place for 30 minutes.
6. After that the (1mL) mixture was add to the HEK293 cell plate and incubated at 10%CO2 and 37℃.
7. The media was changed next day after transfection.
8. After 48 hours of transfection transfected HEK293 cells were selected by growth in G418 (added to media at 1:100 concentrations).
9. After addition of the G418 for 5-7 days, well-defined colonies of HEK293 cells were formed and separated using cloning rings.
10. Colonies were dissociated by addition of few drops of trypsin to each ring, each colony were transferred into separate well of the 24-well plate.
11. After few days protein was secreted into the media.
12. Western blot was done for each colony (well).
13. 60ul of the media of each colony were collected and 20ul of 4X loading buffer were added to each sample followed by boiling the samples to 90℃ for 5-10 minutes.
14. Samples were loaded into 12% acrylamide gel and electrophoresis done at 100 volts for one hour.
15. The proteins were transferred to nitrocellulose membrane at 150 volt for one hour at 4℃.

16. The nitrocellulose membrane was incubated with 5% nonfat milk for one hour to block the binding other nonspecific proteins.
17. The nitrocellulose membrane was incubated with mouse antiflag (Sigma) antibody 1:2500 in (TBST+ 1% nonfat milk) for one hour at room temperature.
18. The membrane was washed 3 times with TBST buffer each time for time for 10 minutes.
19. After that the nitrocellulose membrane were incubated with secondary antibodies (HRP anti-mouse from Alexaflour).
20. The nitrocellulose membrane was washed 3 times with TBST buffer each time for 10 minutes.
21. After that the nitrocellulose membrane was incubated with ECL clarity solution for 2 minutes (Bio-Rad) and the film was exposed to the nitrocellulose membrane in dark room for 1 minutes, 5 minutes and 10 minutes respectively.
22. The correct colonies were expanded to 100cm plate and the WB was repeated after few days for the media to make sure we get the correct molecular weight of the protein together with IgG molecular weight (30 kDa), BDNF (30+13.51), GDNF (30+15.7), NT3 (30+13.63), Noggin (30+23), and SHH-N (30+19).
23. Flasking was done by adding 5 mL trypsin to each 100 cm, centrifugation at 1000 rpm for 5 minutes and resuspension with DMEM. HEK293 cells were re-plated in 125cm flask.
24. The media were changed with DMEM+10%FBS in the first 2 days followed by decreasing the concentration of FBS to 5% at the 3rd day and to 2% in the following days to slow the growth of the HEK293 cells while collecting more protein containing media.
25. The media were collected until the HEK293 cells were detached from the flask.
26. The collected media were centrifuged at 6000 rpm (15,000 x g) for 15 minutes 4°C.

27. A cocktail of protease inhibitor was added in a concentration of 1:100 to maintain our protein from proteolysis and bacterial infection.

Flow Chart 2:
HEK293 Transfection

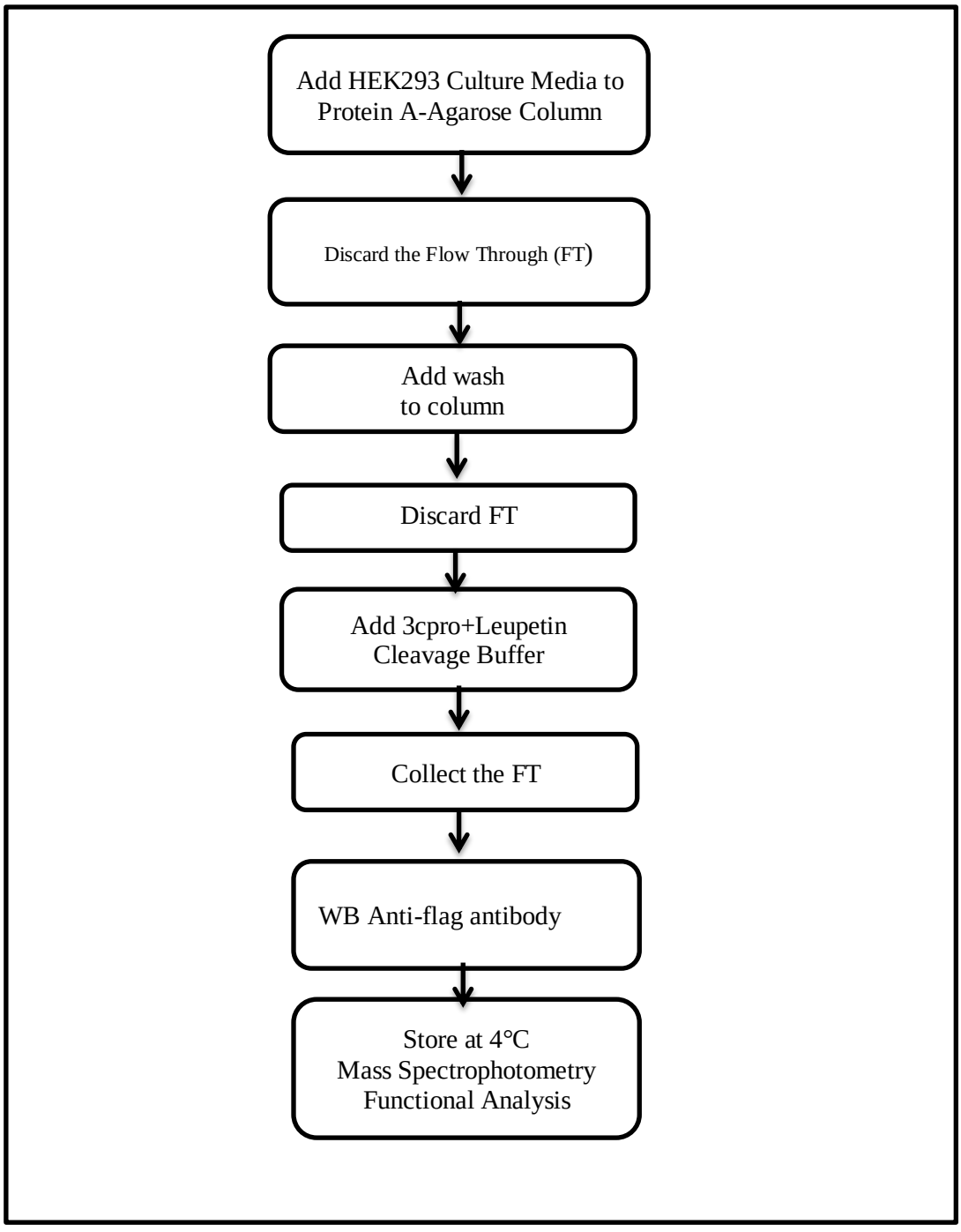


3.3 Protein Purification

Each of the secreted five proteins was purified from the culture medium by the affinity chromatography method using protein A column (Captive-A PriMab affinity resin by RepliGen).

a. To purify the five proteins, 500mL of HEK293 culture medium containing expressed proteins was passed over 2mL protein A agarose column (this step was repeated two times) to make sure that all the proteins in the media are attached to the agarose beads .
b. The flow through was discarded.
c. After chromatography 3cpro and leupeptin containing cleavage buffer were added for 6 hours or overnight at 4℃ to cleave the protein from the agarose beads.
d. The protein was collected in 3K (Amicon) concentrating tubes.
e. The purified protein was concentrated to 0.5-1 mL by centrifugation at 1200 rpm for 10 minutes several times and stored at 4℃.
f. The concentration of the protein was measured by the nanodrop machine (280 A absorption) according to its molecular weight and extinction coefficient).
g. Each protein was confirmed by WB:-sample was loaded onto 12% acrylamide gel and then I transferred them to 0.22 um nitrocellulose membrane. The membrane were blocked for one hour in 5% nonfat milk followed by incubation with primary antibody (mouse anti-flag) overnight at 4℃ then with anti-mouse HRP secondary antibody in TBST buffer for one hour at room temperature. The membrane was developed with ECL clarity solution (Bio-Rad) for 2 minutes.

Flow Chart 3:
Protein Purification



3.4 Functional Characterization

3.4.1 Culturing of iPS Cells:

To confirm that the control iPS cell line 41SE has the characteristics of stem cells, my Ph.D. lab member has stained the iPS cells with stem cell markers antibodies anti (OCT4 and Tra-1-60).

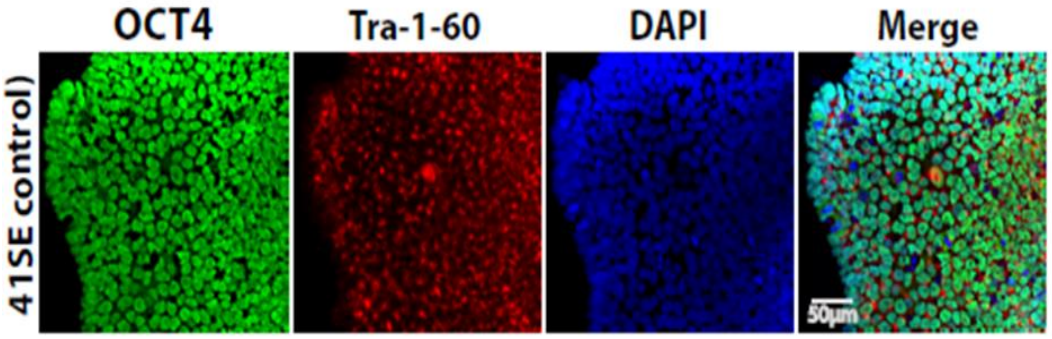


Figure 14: iPS cells express pluripotent markers

Immunohistochemistry staining of the control iPS cell line (41SE) with stem cell markers antibodies: OCT4 (green), Tra-1-60(red) and DAPI (blue) stain of the nuclei. The last image is merging of the 3 stains. By Apoorva Halikere.

3.4.1.1 OCT-4 (Octamer-Binding Transcription Factor4)

(Octamer-binding transcription factor4) this protein is involved in self- renewal of undifferentiated embryonic stem cells. It is use as a marker of undifferentiated stem cells [86, 88].

3.4.1.2 Tra-1-160

200-240 Kda cell surface antigens expressed by ESC and it is act as human stem cell defining antigen [87].

3.4.2 Induction of Neuronal Cells

3.4.2.1 To Test the Effect Of the recombinant (BDNF, GDNF, NT3)

The control 41SE iPS cell line (from RUCDR) was infected with (Ngn2) transcription factor together with rtTA (doxycycline) lentiviral vectors.

3.4.2.1.1 Ngn2 (Neurogenine 2)

Ngn2 is a protein encoded by ngn2 gene that is belong to the subfamily of basic helix-loop-helix (bHLH) transcription factor genes that play an important role in neurogenesis in the brain and spinal cord. It has been found that Ngn2 is sufficient to induce the differentiation of ESCs to glutamatergic (Excitatory neurons) [89].

-Day-1:41SE iPS cells were passaged on matrigel coated 6 well plates 400,000 cells per well.

-Day0: The iPS cells were infected with 3ul/mL Ngn2+4.5ul rtTA per mL mTeser and incubated at 10%CO2 at 37C.

-Day1: Gene expression was induced by addition of Doxicyclin.1:1000 per mL Neurobasal media. (NB)

-Day2 and Day3: The infected cells were selected by the addition of Puromycin1:1000 per mL NB.

- Day4: Mice glia (P0-P2) was plated on matrigel coated coverslips in 24 wells plate, at a density of 50,000 cells/ coverslip in order to promote synapse formation.

-Day 5: The neurons were co-cultured with mouse glia and fed with NB+10%FBS.

-Day 6: Continue changing the media every 4-5 days for three groups of cells with:

* NB+Dox with no added factors. (negative control).

* NB+Dox+ commercial BDNF, GDNF, NT3 1:1000 (conc.100ng/mL) (positive control).

* NB+Dox + recombinant BDNF, GDNF, NT3 1:1000 (conc.100ng/mL) (Test).

-The 3 batches of neurons were fixed at 5 weeks and immunostained for neuronal markers (MAP2, Synapsin, and VGLUT) to compare between the three groups.

3.4.2.2 To Test the Effect of The Purified SHH-N On The Differentiation Of Inhibitory Neurons Induced From IPSCs.

-The same protocol of excitatory neurons induction was done to make inhibitor neurons.
-The iPS cells were infected with (Dlx2), (Ascl1) together with (rtTA) lentiviral vectors.

3.4.2.2.1 Acsl1 (Achaete-scute homolog 1) or MAHSH-1

(Achaete-scute homolog 1) or MAHSH-1 is a member of basic helix loop helix (BHLH) family of transcription factor. It promote cell cycle exit and neuronal differentiation, it promotes the differentiation of embryonic stem cells into inhibitory (GABAergic) neurons through direct effect on the homebox gene Dlx1 and Dlx2 [90].

3.4.2.2.2 Dlx2 (Distal less homeobox gene)

Dlx2 (Distal less homeobox gene): is a family of homeodomain transcription factors which are related to the Drosophila distal-less (Dll) gene. It promotes induction of inhibitory neurons from ESCs and iPS cells [91].

-Make 3 groups of neurons:

(control): NB+ commercial neurotrophins 1:1000+Dox1:1000)

(100 ng/mL SHH-N): NB+ commercial neurotrophins 1:1000+Dox1:1000) + 100ng/mL recombinant SHH-N starting from day 6 of differentiation.

(200ng/mL SHH-N): NB+ commercial neurotrophins 1:1000+Dox1:1000) + 200ng/mL recombinant SHH-N starting from day 6 of differentiation.

-The 3 batches of neurons were fixed at 5 weeks and immune-stained for neuronal markers (MAP2, Synapsin, and VGAT) to compare between the three groups.

3.4.2.3 To Test the Effect of My Purified Noggin on the Differentiation of Dopaminergic Neurons Induced From IPSC

-I followed the same protocol of excitatory neurons induction to make dopaminergic neurons.

-The iPS cells were infected with (Lmx1), (Ascl1), (Nurr1) (rtTA) on day-1, followed by (Foxa1), (Pitx3), (En1) and rtTA together with lentiviral vectors.

3.4.2.3.1 Lmx1a

Lim homeobox transcription factor 1, alpha, it plays an important role in human and mice midbrain dopaminergic neuron differentiation from embryonic stem cell [60].

3.4.2.3.2 Nurr1

Nuclear receptor related 1 protein (NURR1), is a member of the nuclear receptor family of intracellular transcription factors. It plays an important role in the differentiation and maintenance of the dopaminergic system of the brain [93]

3.4.2.3.3 Foxa1

Fork head box protein A1, it is a member of fork head class of DNA binding protein. Together with Foxa2 they regulate the development of midbrain dopaminergic neurons via the regulation of expression of engrailed 1 and Nurr1 in the immature neurons [94].

3.4.2.3.4 En1

Engrailed 1 and 2 are homeobox protein essential for the late maturation of midbrain dopaminergic neurons [95].

3.4.2.3.5 Pitx3

Pituitary homeobox protein 3 it is essential for the differentiation and maintenance and long term survival of dopaminergic neurons [96].

-Two groups of neurons were fed by:

1. NB+ Commercial neurotrophins (1:1000) +Dox (1:1000)

2.NB+ Commercial neurotrophins (1:1000) +Dox (1:1000) + recombinant Noggin (500ng/mL)

-The 3 batches of neurons were fixed at 5 weeks and immune-stained for neuronal markers (MAP2, Synapsin, and TH) in order to compare between the two groups.

3.4.3 Electrophysiology

Whole cell clamp recording of the three groups of inhibitory neurons at the fifth week of differentiation to compare the differences in properties spontaneous current and action potential of the three groups. (This recording is done by my Ph.D. colleague Apoorva Halikeree).

Action potentials were recorded with current-clamp whole cell configuration .The solution of the pipette that is used for the current clamp experiments contains (in mM): 123 K-gluconate, 10 KCl, 1MgCl2, 10 HEPES, 1 EGTA, 0.1 CaCl2, K2ATP, 0.2 Na4GTP 0.4, and glucose, PH adjusted to 7.2 with KOH. Membrane potentials were kept around -65 to -70 mV, and step currents were injected to induce action potentials. For evaluation of synaptic functional evaluation, the internal solution consist of (in mM); CsCl 135, HEPES 10,EGTA 1, Mg-ATP 4, Na4GTP 0.4, and QX-314 10,PH7.4 .The bath solution contained (in mM): NaCl 140,KCl 5, CaCl2 2,MgCl2 2, HEPES 10, and glucose 10, Ph7.4[16].

A neuron intended for postsynaptic recordings was patched with pipettes that were pulled from borosilicate glass capillary tubes (Warner Instruments, Cat# 64-0793) using a PP830 pipette puller (Narishige).). The frequency, duration, and magnitude of the extracellular stimulus were controlled with a Model 2100 Isolated Pulse Stimulator (A-M Systems, Inc.) synchronized with Clampex 9 data acquisition software (Molecular Devices) [97].

3.4.4 Immunostaining

1) The cover slips were washed once with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature.
2) The coverslips were washed once with PBS and permeated by 0.02% Triton for 15 minutes at room temperature.
3) After that the coverslips were blocked with 2% BSA1% Goat serum for 1 hour at room temperature.
4) Incubation of the cover slips with the following primary antibodies for the neuronal markers:

   *Mouse anti MAP2 (sigma 1:500), and, rabbit anti Synapsin (E028 1:1000) (for Inhibitory, excitatory and dopaminergic neurons)

   *Rabbit anti-VGAT (Millipore 1:1000) and anti GAD67 (Sigma 1:1000) for inhibitory neurons.

   *Mouse anti VGLUT (Millipore 1:1000) for excitatory neurons.

   * Mouse anti TH (Millipore 1:1000) for dopaminergic neurons.

These antibodies were added to the corresponding neurons coverslips in blocking buffer and incubated at 4℃ overnight.

5) The coverslips were washed 3 times with PBS.
6) The coverslip were incubate with the secondary antibodies (Alexa flour) 488 anti-mouse and (Invitrogen) 546 anti-rabbit for 30 minutes at room temperature.
7) The coverslips were washed three times with PBS.
8) The coverslips were washed once with sterilized water.
9) The coverslips were dried with Kim wipes and mounted on the glass slide using Flourosheild mounting media (Sigma) that contain also DAPI to stain the nuclei.
10) The slides were labeled and stored at 4°C.

3.4.4.1 Microtubule-associated protein 2 (MAP2)

Microtubules are 25nm diameter protein rods found in most kinds of eukaryotic cells .It has many isoforms.MAP2 isoforms are expressed only in neuronal cells. It stabilizes the microtubules growth by cross linking them with intermediate filaments. It plays an important role in stabilization of dendrites and determination of neuronal shape during neuronal development. It also regulates the balance between the rigidity and the plasticity of neuronal processes [98]. It is used as neuronal marker; it is expressed in the neuron shortly after the expression of neuron-specific tubulin isoform βIII).

3.4.4.2 Synapsin

Synapsin is neuron specific phosphoprotein that is located on the cytoplasmic surface of synaptic vesicles in the majority of nerves terminal. It plays an essential role in synaptic formation, stabilization and modulation of synaptic function [99]. Its phosphorylation by cyclic AMP and two calmodulin/calcium dependent protein kinases make it dissociate from the synaptic vesicle which become destabilized ,and this facilitate the release of neurotransmitter [100].

3.4.4.3 Vesicular GABA Transporter (VGAT)

Vesicular GABA transporter is highly concentrated in GABAergic neurons nerve ending. It is responsible for the uptake and storage of GABA and glycine neurotransmitter. It is the best known marker for inhibitory neurons. GABA is a

neurotransmitter that is synthesized from glutamic acid by the enzyme called glutamate decarboxylase (GAD) and is accumulated in the synaptic vesicles by VGAT [68, 69].

3.4.4.4 Vesicular Glutamate Transporter (VGLUT)

Vesicular glutamate transporter is a protein that functions in the uptake and storage of L-glutamate into the synaptic vesicles of the excitatory neurons [103]. Glutamate is the main neurotransmitter of excitatory neurons that is synthesized from glutamine [104].

3.4.4.5 Tyrosine Hydroxylase (TH)

Tyrosine Hydroxylase is the enzyme that converts the amino acid L-tyrosine to 3, 4 L-dihydroxyphenylalanine (L-Dopa) which is the precursor for dopamine, norepinephrine and epinephrine. It is highly localized in the cell bodies and axons of dopaminergic neurons [105].

3.4.4.6 DAPI (4', 6-diamidino-2-phenylindole)

4', 6-diamidino-2-phenylindole is a blue fluorescent stain that emits when it bind to the AT rich region of the DNA. It crosses the plasma membrane easily and used to stain the nuclei of living and fixed tissues [106].

3.4.5 Synaptic protein staining analysis

Synaptic protein staining was performed on five-week-old Ips induced excitatory neurons co-cultured with mouse glia.

To calculate the ratio of synaptic protein to MAP2AB-Positive dendrites, confocal image were taken at 360X magnification, using NIH imageJ, images were thresholded and integrated pixel density was determined for each image. Integrated pixel density is the product of (measure in square pixel) and means gray value (the sum of gray values of all the pixels in the selection divided by the number of pixels).

Chapter 4: Results

4.1 BDNF, GDNF, NT3, Noggin and SHH-N: Cloning, Transfection and Protein Purification

In order to obtain recombinant proteins, the cDNA of each of the five proteins BDNF, GDNF, NT3, Noggin and SHH-N were inserted into XbaI and NotI region of pCMV6-XL4 vector. Next I transfected each of the five constructs into HEK 293 cells. When the HEK293 started to express each of the five recombinant proteins into the media as a soluble human-IgG fusion protein, I separated several colonies of HEK293 cells and did Western blots for each of them using anti IgG antibody. When I made sure that I have the correct protein being expressed in the media I expanded each of the correct colonies into bigger flasks and started to collect the media. After that I purified the proteins by affinity chromatography method, the IgG portion of each of the five proteins bound to protein A in the beads (resin). I eluted the protein with special buffer to separate the protein from the IgG portion. To confirm that I have the correct protein I performed Western blot using anti-flag antibody. The five proteins appeared in reducing SDS-PAGE gels as a broad band with an apparent molecular weight (MW): BDNF: 13.5 kDa, GDNF: 15.7 kDa, NT3:13.6 kDa. Noggin: 23.5 KDa, SHH-N: 19.6 kDa (Fig.15).

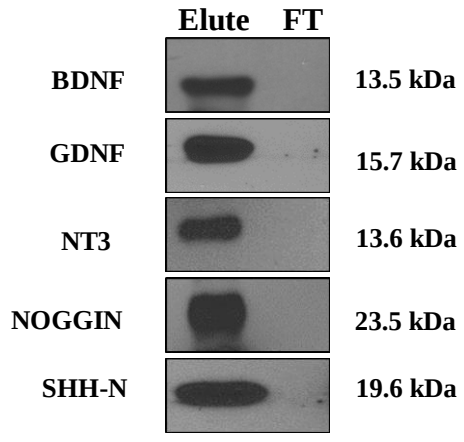


Figure 15: Western blot of the purified proteins

BDNF and the flow through (FT), molecular weight (MW): 13.5 kDa. GDNF and FT, W: 15.7 kDa, NT3 and FT, MW: 13.6 kDa, Noggin and FT, MW : 23.5 kDa, SHH-N and FT, MW: 19.6 Kda.

4.2 Induction of Excitatory human (iNs):

I converted the iPS cells into excitatory neurons by forced expression of one transcription factor Ngn2, using lentiviral delivery for constitutive expression of rtTA, and tetracycline inducible expression of this gene under the control of tet-O-promotor. I also co-expressed puromycin resistance gene with Ngn2 to select for cells expressing the transcription factor. The iPS cells were plated at day-2, infected with lentivirus on day-1, Ngn2 was induced with doxycycline on day 0. A 24 hour puromycin selection period was started on day 1. The mouse glia was plated on day 4 and the neurons were dissociated and co-cultured with primary mouse glia on day 5 of neuronal differentiation. The growth factors were added at day 6.

Forced Ngn2 expression iPS cells into neuron like-cells in less than one week and produced mature neuronal morphological appearance in less than two weeks.

At 5 weeks of neuronal differentiation staining of the excitatory neuron showed the expression of neuronal marker MAP2 that stains neuronal cell bodies and dendrites, and Synapsin which stains the synaptic vesicles protein at neuronal synapses (Fig.16).

The induced excitatory neurons also express Glutamatergic neurons marker VGLUT (Fig.17).

The three conditions: Negative control, Positive control (to which I added the commercial BDNF, GDNF, NT3 starting from day 6 of neuronal differentiation) and Test (to which I added the recombinant BDNF, GDNF, NT3 starting from day 6 of neuronal differentiation) showed no difference in expression of neuronal markers MAP2, Synapsin and VGLUT.

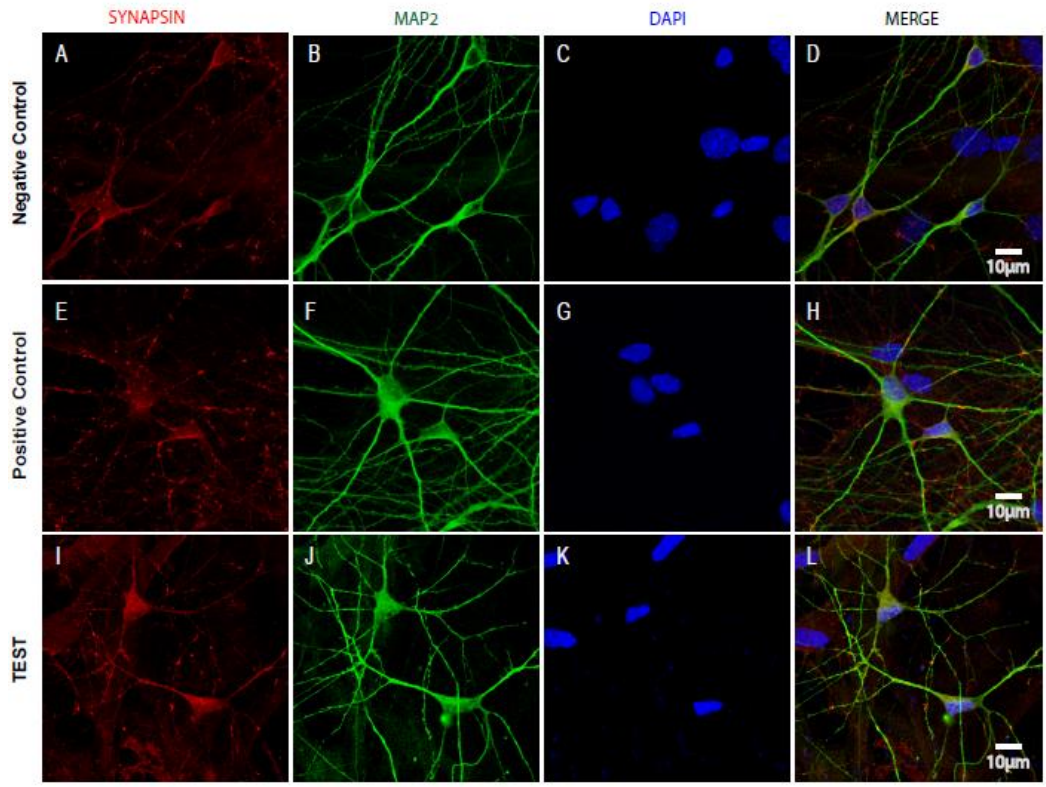


Figure 16:Immunohistochemistry staining of iPS induced excitatory neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ngn2, rtTA).

(A,E,I) shows Synapsin staining of the three conditions (A)Negative control : no Neurotophins were added during neuronal differentiation (B) Positive control: commercial neurotrophins (BDNF,GDNF,NT3) were added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(I) Test: the recombinant neurotrophins (BDNF,GDNF,NT3) were added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(B,F,J) show MAP 2 staining of the three conditions
(Negative control, Positive control, Test) respectively.(C,G,K) shows DAPI staining of the three conditions(Negative control, Positive control, Test) respectively.
(D,H,L) is the merge of the three stains.

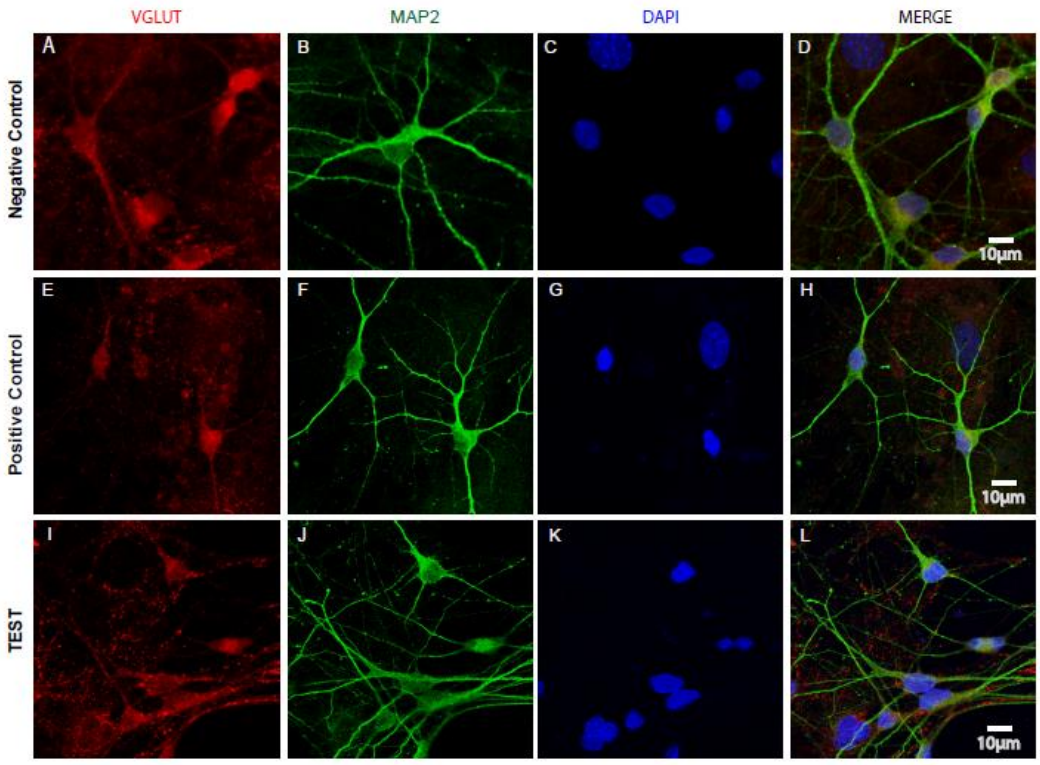


Figure 17: Immunohistochemistry staining of iPS induced excitatory neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ngn2, rtTA).

(A,E.I) shows VGLUT staining of the three conditions (A)Negative control; no neurotophins were added during neuronal differentiation(B) Positive control: commercial neurotrophins (BDNF,GDNF, and NT3) in 100ng/mL concentration starting from day 6 of neuronal differentiation.(I) Test: the recombinant neurotrophins (BDNF,GDNF,NT3) where added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(B,F,J) show MAP2 staining of the three conditions (Negative control, Positive control, Test) respectively.(C,G,K) shows DAPI staining of the three conditions (Negative control, Positive control, Test) respectively.(D,H,L) is the merge of the three stains.

Next I calculated the synapsin puncta per micrometer length of the dendrite which give us an estimate of the difference in number of synapses of the induced excitatory neurons among the three groups. (Fig.18) shows that the Test condition has higher number of synapses in comparison with other two groups (Positive control and Negative control). Positive control seems to have higher number of synapses than Negative control as well.

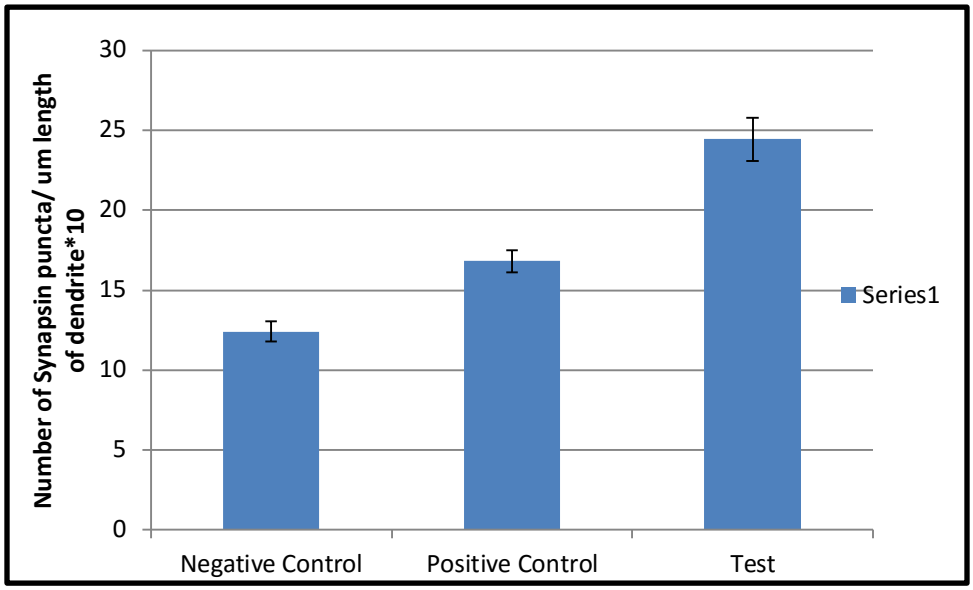


Figure 18: synapsin quantification of iPS Induced Excitatory neurons for Synapsin-MAP2 immune-stained coverslips fixed at 5weeks of differentiation.

(Negative control): no growth factors were added. (Positive control): Commercial neurotrophins (BDNF, GDNF, and NT3) were added in100ug/mL concentration starting at day 6 of neuronal differentiation. (Test): The recombinant neurotrophins (BDNF, GDNF, and NT3) were added in 100ug/mL concentration starting at day 6 of neuronal differentiation.

4.3 Induction of Dopaminergic human (iNs)

I converted the iPS cells into dopaminergic neurons by forced expression of the six transcription factors Ascl1, Nurr1, Lmx1, Foxa1, Pitx3, En1, using lentiviral delivery for constitutive expression of rtTA, and tetracycline inducible expression of those genes under the control of tet-O-promotor. I also co-expressed puromycin resistance gene with Ascl1, Nurr1, Lmx1, Foxa1, Pitx3, and En1 to select for cells expressing these transcription factor. The iPS cells were plated at day-2, infected with lentivirus Ascl1, Nurr1, Lmx1 on day-1, expression were induced with doxycycline on day 0. A 24 hour puromycin selection period was started on day 1, followed by infection with the lentiviruses Foxa1, Pitx3, En1 on day 3. The mouse glia was plated on day 4 and the neurons were dissociated and co-cultured with primary mouse glia on day 5 of neuronal differentiation. The recombinant Noggin was added in a concentration of 500ng/mL starting at day 6.

Forced Ascl1, Nurr1, Lmx1, Foxa1, Pitx3, En1expression converted iPS cells into neuron like-cells in less than one week and produced mature neuronal morphological appearance in less than two weeks.

I next stained the dopaminergic neurons after fixing the cells at week five of neuronal differentiation. The staining showed that the induced dopaminergic neurons express pan-neuronal markers MAP2 which stains neuronal dendrites and cell bodies and Synapsin which stains synaptic vesicles protein at neuronal synapses. (Fig.19)

The induced dopaminergic neurons also expressed dopaminergic neurons marker TH. (Fig.20)

There are no distinguishable differences in the staining of MAP2, Synapsin and TH between the control group and the test group to which I applied Noggin 500ng/mL starting from day 6 of neuronal differentiation.

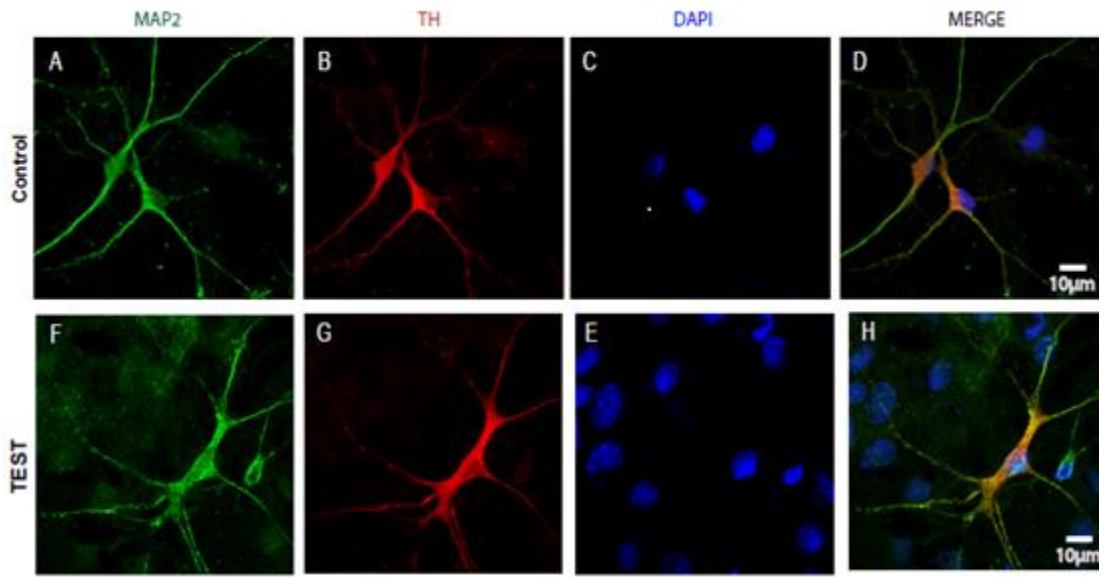


Figure 19:Immunohistochemistry staining of iPS induced dopaminergic neurons

(A,F) shows MAP2 staining of the two conditions (A) Control:; no Noggin added during neuronal differentiation(F) Test: recombinant Noggin were added in 500ng/mL concentration starting from day 6 of neuronal differentiation.(B,G) show TH staining of the two conditions (Control, Test) repecively. (C,E) shows DAPI staining staining of the two conditions (Control, Test) respectively.(D,H,) is the merge of the two stains.

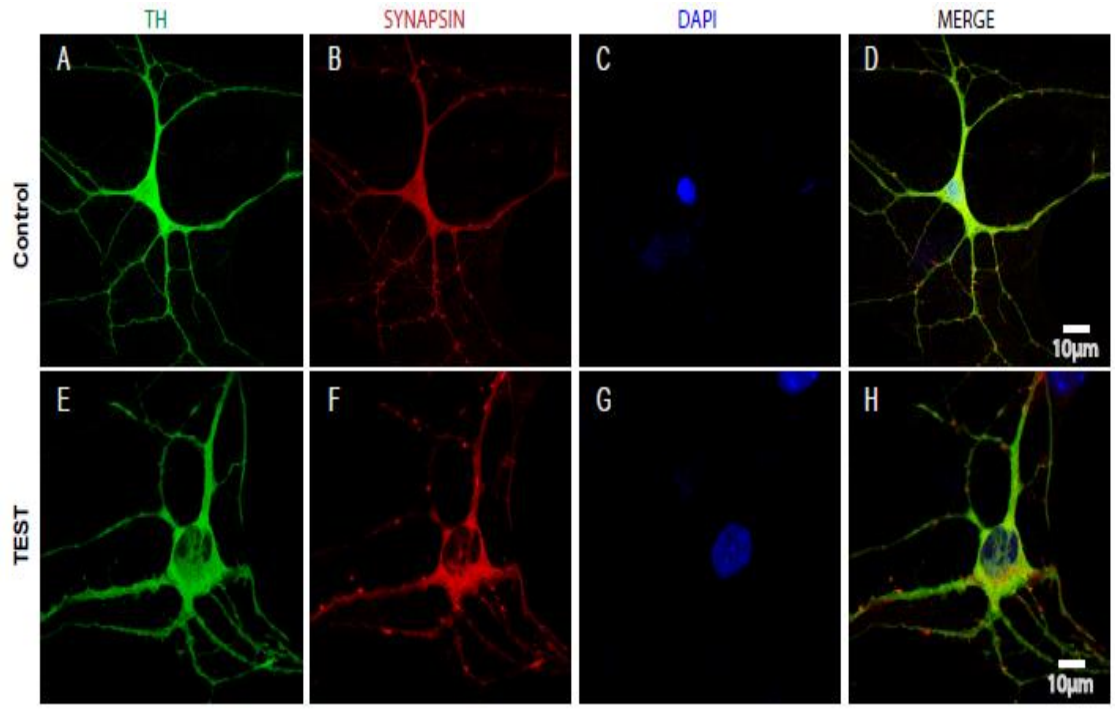


Figure 20:Immunohistochemistry staining of IPSC induced dopaminergic neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ascl 1, Lmx1, Nurr1, Pitx3, Foxa1, and rtTA).

(A,E) shows TH staining of the two conditions (A) Control: no Noggin were added during neuronal differentiation(F) Test: recombinant Noggin were added in 500ng/mL concentration starting from day 6 of neuronal differentiation.(B,G) show Synapsin staining of the two conditions (Control, Test) respectively. (C,E) shows DAPI staining staining of the two conditions (Control, Test) respectively. (D,H,) is the merge of the two stains.

4.4 Induction of Inhibitory human (iNs)

I converted the iPS cells into inhibitory neurons by forced expression of the two transcription factors Ascl1, Dlx2, using lentiviral delivery for constitutive expression of rtTA, and tetracycline inducible expression of those genes under the control of tet-O-promotor. I also co-expressed puromycin resistance gene with Ascl1 and Dlx2 to select for cells expressing the two transcription factor. The iPS cells were plated at day-2, infected with lentivirus on day-1, Ascl1 and Dlx2 expression was induced with doxycycline (Dox) on day 0. A 24 hour puromycin selection period was started on day 1. The mouse glia was plated on day 4 and the neurons were dissociated and co-cultured with primary mouse glia on day 5 of neuronal differentiation. Application of the recombinant SHH-N 100ng/mL or 200ng/mL was started on day 6. Forced Ascl1 and

Dlx2 expression iPS cells into neuron like-cells in less than one week and produced mature neuronal morphological appearance in less than two weeks.

At 5 weeks of differentiation staining of the induced inhibitory neurons showed that they expressed mature neuronal markers MAP2 which stains the neuronal cell bodies and dendrites and Synapsin which stains the synaptic vesicle protein at neuronal synapses (Fig.21) .

The induced inhibitory neurons also expressed GABAergic neurons marker (VGAT) (Fig.22) and GAD67 (Fig.23).

There is no obvious differences in the staining of MAP2,Synapsin,VGAT and GAD67 among the three conditions of induced inhibitory neurons; Control, 100ng/mL SHH-N (to which I added 100ng/mL of the recombinant SHH-N starting from day 6 of neuronal differentiation), 200ng/mL SHH-N (to which I added 200ng/mL starting from day 6 of neuronal differentiation).

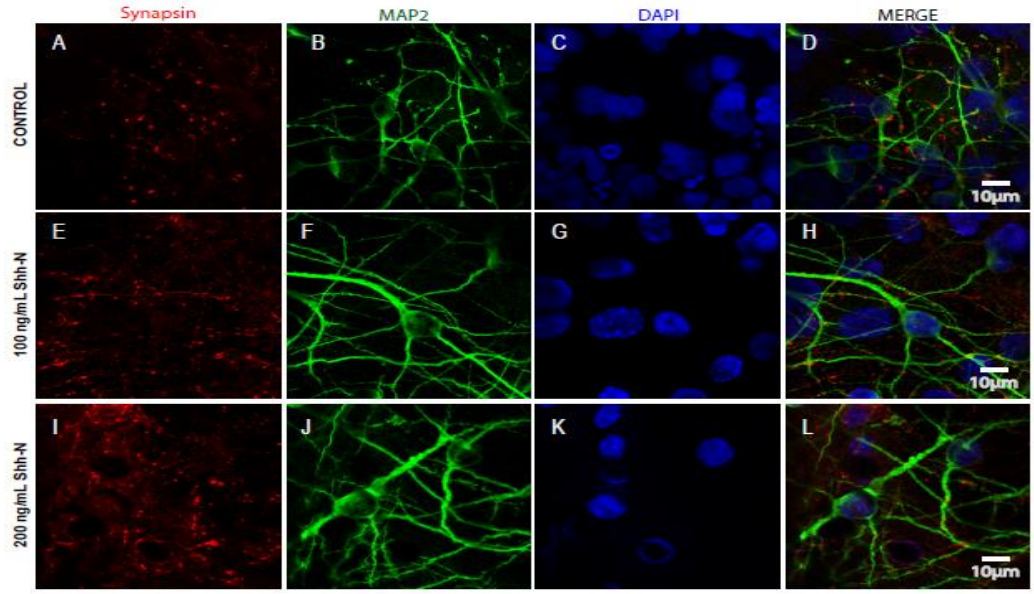


Figure 21: Immunohistochemistry staining of iPS induced Inhibitory neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ascl1, Dlx2, and rtTA).

(A,E,I) shows Synapsin staining of the three conditions (A) Control: no SHH-N were added, (B) SHH-N were added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(I) SHH-N were added in 200ng/mL concentration starting from day 6 of neuronal differentiation.(B,F,J) show MAP2 staining of the three conditions (Control,100ng/mL,200ng/mL) respectively.(C,G,K) shows DAPI staining of the three conditions (Control,100ng/mL, and 200ng/mL) respectively. (D,H,L) is the merge of the three stains.

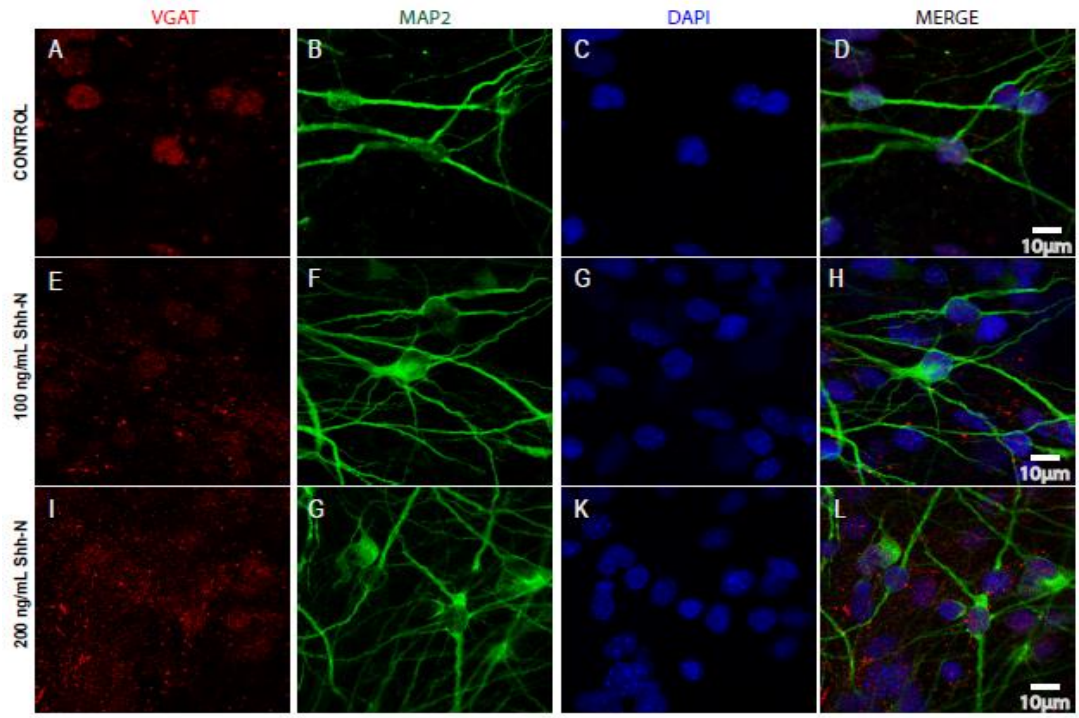


Figure 22: Immunohistochemistry staining of iPS induced Inhibitory neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ascl1, Dlx2, and rtTA).

(A,E,I) shows VGAT staining of the three conditions (A) Control: no SHH-N were added, (B) SHH-N were added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(I) SHH-N were added in 200ng/mL concentration starting from day 6 of neuronal differentiation.(B,F,J) show MAP2 staining of the three conditions (Control,100ng/mL,200ng/mL) respectively. (C,G,K) shows DAPI staining of the three conditions (Control,100ng/mL, and 200ng/mL) respectively.(D,H,L) is the merge of the three stains.

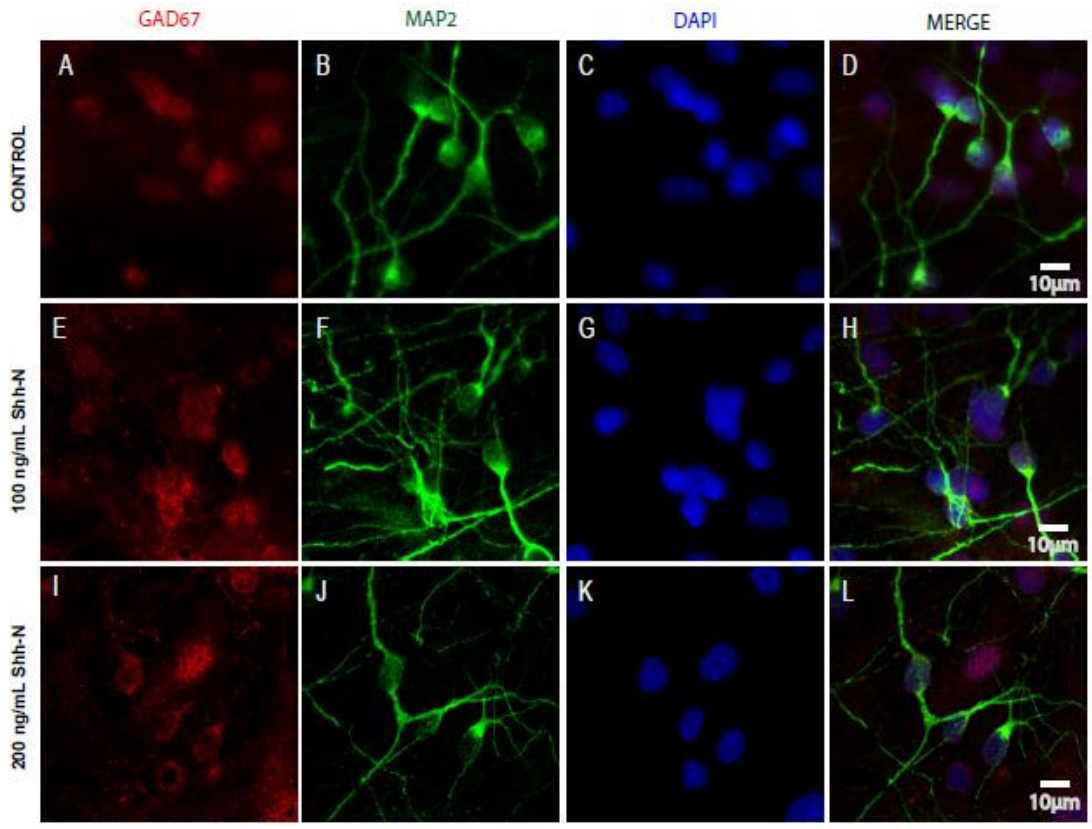


Figure 23: Immunohistochemistry staining of iPS induced Inhibitory neurons after 5 weeks of infection with specific transcription factors lentiviral vectors (Ascl1, Dlx2, and rtTA).

((A,E,I) shows GAD67 staining of the three conditions (A) control: no SHH-N were added, (B) recombinant SHH-N were added in 100ng/mL concentration starting from day 6 of neuronal differentiation.(I) recombinant SHH-N were added in 200ng/mL concentration starting from day 6 of neuronal differentiation.(B,F,J) show MAP2 staining of the three conditions (Control,100ng/mL, and 200ng/mL) respectively.(C,G,K) shows DAPI staining of the three conditions (Control,100ng/mL, and 200ng/mL) respectively.(D,H,L) is the merge of the three stains.

4.4.1 Electrophysiological recording of inhibitory iNs

Next I tried to assess whether the iPS cells induce inhibitory neurons exhibit an active membrane properties. Whole-cell patch clamp recording of inhibitory neurons was done after 5 weeks of culture following induction with doxycycline by my Ph.D. colleague Apoorva Hailikere.

The iPS induced inhibitory neurons were able to form functional synapses and fires spontaneous action potential. Extracellular stimulation evoked inhibitory post synaptic current (IPSC).

When I quantify the number of neurons that have spontaneous action potential, there was no distinguishable difference observed among the three groups (Control, 100 ng/mL SHH-N, and 200ng/mL SHH-N (Fig. 24).

Next I quantified the number of neurons that show inhibitory post synaptic current (IPSC) and there were no obvious differences among the three groups (Fig.25).

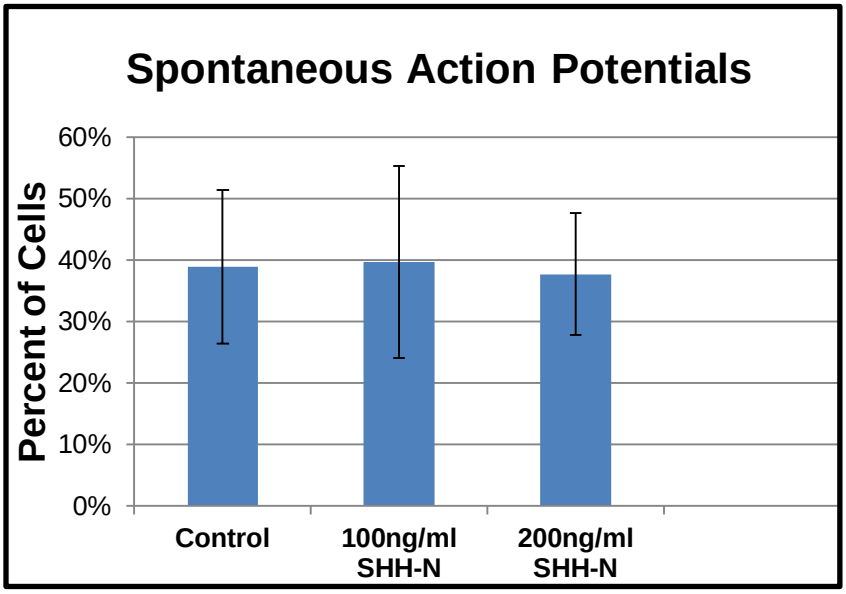


Figure 24: Percentage of induced inhibitory neurons that show spontaneous action potential.

Whole-cell-patch clamp recording (By Apoorva Halikere).It shows spontaneous action potential 5 weeks after infection with lentiviral transcription factors lentivirus. The result is plotted for the three conditions (Control: no SHH-N added, 100ng/mL of the recombinant SHH-N added, and 200ng/mL of the recombinant SHH-N added starting from day 6 of neuronal differentiation).

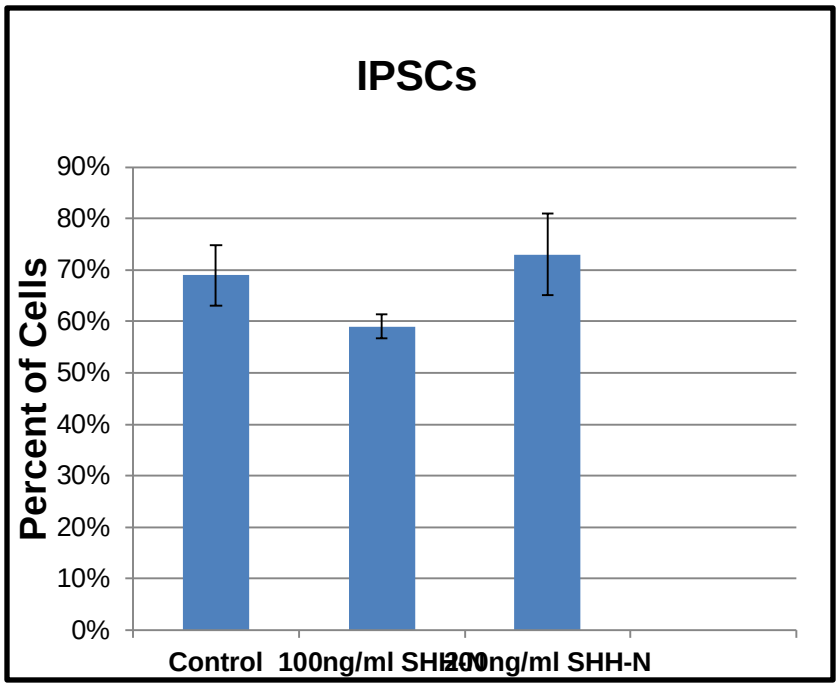


Figure 25: Percentage of induced inhibitory neurons that have inhibitory postsynaptic current after evoked stimulation.

Whole-cell-patch clamp recording (By Apoorva Halikere). At 5 weeks after infection with lentiviral transcription factors. The result is plotted for the three conditions (control: no SHH-N added, 100ng/mL of the recombinant SHH-N added, and 200ng/mL of the recombinant SHH-N added starting from day 6 of neuronal differentiation).

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Chapter 5: Discussion and Future Directions

5.1 Discussion

Production of recombinant proteins by cultivated mammalian cells has become an important source of factors (hormones, immunoglobulin, growth factors, etc.) that can be beneficial for clinical application and research purposes.

The aim of this study was to create a stable cell line for the production of five proteins that play a role of essence in specification, growth and maintenance of specific neuronal subpopulations during development and in adult life.

Among these proteins are BDNF, GDNF, NT3, NOGGIN and SHH-N. The obtainment of a cell line as a source for large scale production of these proteins would represent a unique tool for the low cost generation of different neuronal subtypes.

This study has been focused on the construction of five expression vectors carrying respectively BDNF, GDNF, NT3, NOGGIN and SHH-N genes followed by transfection of HEK293 cells and by the purification of the recombinant proteins from the supernatant. Upon validation of the proteins according to both qualitative and functional standard criteria, I have shown a novel, effective and low cost method to generate -ready to use- growth factors and neurotrophins.

In particular, I analyzed three distinct subtypes of neurons; Excitatory (glutamatergic neurons), Inhibitory (GABAergic neurons) and Dopaminergic neurons.

Most importantly, my study has shown that these recombinant proteins can be efficiently used to facilitate the reprogramming of iPS cells into neurons, with a considerable advantage over viral vectors and related risk of chromosomal integration and malignancy formation.

First, to assay their role in neuronal differentiation, I applied three of those recombinant proteins (BDNF, GDNF, and NT3) during the lentiviral reprogramming of iPS cells to excitatory (glutamatergic) neurons.

Excitatory neurons comprise 80% of the cerebral cortex neurons and they represent the projection neurons that transmit information to other cortical and subcortical regions of the brain. Disturbance of the function of excitatory neurons has been shown in several

neurological diseases such as Alzheimer disease, Rett syndrome, Huntington disease and others. In this view, optimizing an efficient method for generation of excitatory neurons is necessary for better understanding and management of these diseases.

Neurotrophins (BDNF, NT3) are known for their ability to potentiate excitatory (glutamatergic) synaptic transmission through their binding to Trk receptor. Activation of Trk receptor increases the release of glutamate neurotransmitter from the presynaptic membrane. In addition, BDNF enhances phosphorylation of glutamate receptor (NMDA); thus potentiating the post synaptic effect of glutamate [47, 48].

Furthermore, GDNF is potent neurotrophic factor that has neuroprotective effect and can enhance neurotransmitter release via its effect on calcium currents [54]. GDNF also regulates cortico-striatal glutamatergic pathways through co-activation of adenosine A (2A) receptors [107].

To determine whether recombinant neurotrophic factors can promote differentiation of iPS to excitatory neuron , I used different approaches and compared the induced neurons obtained with recombinant proteins to those obtained in factor-free medium (negative control) or in commercial factors-containing medium (positive control). Immunostaining of neurons at 5 weeks showed that excitatory neurons that are generated from iPS cells are expressing the neuronal marker MAP2, synapsin, in addition to the excitatory neuronal maker VGLUT.

No obvious morphological differences were detectable with respect to positive control neurons, suggesting that the recombinant proteins do not alter the differentiation process with respect to commercial factors.

Quantification of synapsin puncta per unit length of the dendrite showed that addition of the recombinant BDNF, GDNF, and NT3 (Test) to the induced excitatory neurons has higher number of synapsin when compared to both the control conditions (negative control and positive control). Because synapsin is a key protein involved in synaptic vesicle release, greater synapsin puncta per unit dendrite is indicative of greater functional connectivity between iNs.

This finding suggests that the recombinant (BDNF, GDNF, and NT3) might be more effective than the commercial factors.

Further analysis is needed such as mass spectrometry to compare the recombinant proteins amino acid sequence with the correct protein database. Another way to test the functionality of the protein is by immunoprecipitation or affinity chromatography method to detect the interaction between each of the five recombinant proteins and their specific receptor.

The experiment for additional batches is in progress, in order to provide a quantitative evaluation of the consistency of the method.

Previous evidence from Zeng et al (2010), Espuny et al. (2013) and Zhang et al have shown the generation of glutamatergic neurons (Pyramidal neurons) from ESC and iPS cells , that were functional, expressing neuronal markers and able to integrate upon transplantation into mouse brain [108,109,110].

Second, I have tested the effect of the recombinant SHH-N on the induction of inhibitory (GABAergic neurons) from iPS cells.

Inhibitory neurons constitute the 20% of the cerebral cortex. They are crucial for the regulation of firing, probability and timing of action potential [42] by modulating and synchronizing the activity of excitatory neurons. Improper function of GABAergic neurons is the underlying reason for many neuropsychiatric disorders such as Schizophrenia, Epilepsy, and Autism related disorders and others.

During brain development, SHH enhances the conversion of primitive dorsal precursors to ventral progenitors, which will give rise to GABAergic neurons via the activation of continuous expression of Nkx1.2 [111, 30].

In this study, I have shown by immunostaining that the inhibitory neurons induced from iPS cells through the addition of recombinant SHH do express the mature neuronal markers MAP2 and synapsin in addition to the subtype-specific markers of inhibitory neurons VGAT and GAD67, suggesting that recombinant SHH-N is able to exert *in vitro* the same function as *in vivo*.

While the role of sonic hedgehog in inhibitory neuron differentiation is clear, there are no clear differences among the three groups of inhibitory neurons (control, 100ng/mL SHH-N, 200ng/mL SHH-N).

Electrophysiological recording at week 5 of neurons in all the three groups of induced inhibitory neurons revealed that they have spontaneous postsynaptic currents. They also fire inhibitory evoked postsynaptic current upon evoked stimulation. The percentage of cells that have spontaneous action potential is almost similar among the three groups (control, 100ng/mL SHH-N, 200ng/mL SHH-N).

The same result can be seen regarding the percentage of neurons that fires inhibitory postsynaptic current upon evoked stimulation.

This result may indicate that the recombinant SHH-N might be not quite functional and require further processing. There might be a problem related to protein folding, post-translational modification, eluting buffer composition or storage conditions. All these conditions may affect the function of the protein even if it has the correct band size on SDS-gel.

Inhibitory neurons have been induced successfully from ESC by Maroof et al (2013) [73], Nicholas et al. (2013) [112].

Maroof et al. [73] showed that the fate of induced neurons produced from ESC is determined by the timing of SHH activation. He proved that late activation of SHH (at 10-18days of neuronal induction) lead to generation of GABAergic neurons with (MGE) like features.

In this study I have applied the recombinant SHH-N at day 6 of neuronal induction, so it will be relevant to repeat the induction of inhibitory neurons with late application of SHH-N.

Lixiang Ma and his colleagues [110] have generated GABAergic neurons from ESC using different concentration of SHH starting from 0-500 ng/mL SHH starting from day 12 of neuronal differential differentiation. They found that 200ng/mL SHH has produced optimal expression of medium spiny GABA neuron markers, and transplantation of these neurons in the brain of Huntington's disease mouse model has corrected the locomotors deficit.

Better tuning the time and dosage of sonic hedgehog application will greatly facilitate neuronal maturation and subtype specificity.

These previous finding proved that the effect of SHH on GABAergic neuronal specification depends on the dose and timing of SHH-N application.

After verification of the functionality of the recombinant SHH by mass spectrometry and other protein processing assay, we can manipulate the dose and timing of SHH during the process of GABAergic neurons induction from iPS cells.

Third, I tested the effect of the recombinant Noggin on dopaminergic neuron induction from iPS cells starting from day 6 of neuronal differentiation. Many studies have been performed to generate functional dopaminergic neurons from ESC, astrocytes, iPS and fibroblast using transcription factors or growth factor. [34, 83, 15,113]

The exploitation of Noggin as a fate-determining factor in generation of dopaminergic neurons from iPS cells is a novel idea that deserves attention but needs further investigation.

Midbrain dopaminergic neurons are the main source of dopamine in mammalian brain. Dopamine is essential for control of voluntary movement and emotion based behavior. Dopaminergic neurons deficiency results in serious illness known as Parkinson disease or less serious anxiety disorders [84].

Noggin has been proved to play an important role in dopaminergic neuron differentiation from embryonic stem cells [80, 81].

To assay the role of Noggin in the lentiviral induction of iPS cells to neurons, I used the recombinant Noggin for dopaminergic neuron differentiation. Immunostaining of 5 weeks induced dopaminergic neurons showed that neurons express the dopaminergic marker TH in addition to mature neuronal markers MAP2 and synapsin.

There are no apparent morphological differences between the control and the test (500ng/mL) groups. This is the only study that I did for this type of induced neurons due to the time limitation.

Overall, in this project, I have purified recombinant small factors, i.e. BDNF, GDNF, NT3, Noggin and SHH-N that can promote the maturation of neurons and support synaptogenesis. The results shown that these factors are functional thus can be used for supporting neuronal differentiation.

5.2 Future Directions

- **Verification of the structural and functional identity of the five recombinant proteins is required for further application in the process of neuronal induction.**
- **Repetition of the experiments with modification of differentiation protocol and try to eliminate the viral method for delivery of transcription factors.**
- **Perform more thorough analysis for the induced neurons such as genetic profile comparison by QT-PCR, patch clamp recording, neuronal and synapses quantification between the control and the test conditions.**
- **Try to test the in vivo activity of the induced neurons and their ability to form synapses with the surrounding neuronal tissues.**
- **Application of these factors on generation of neurons from iPS cells derived from animal models with neurological diseases.**

With the use of recombinant proteins, we can facilitate the production of subtype specific neurons to help regenerate brain tissue of specific brain regions.

Using the technology of purified human proteins, we can achieve more far reaching goals as well. For example, this technique can be used to identify the function of novel proteins involved in neuronal function and development.

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