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Neural Transdifferentiation of embryonic like stem cells by lithium chloride

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ABSTRACT

Spermatogonial stem cells (SSCs) because of its ability to be reprogrammed into embryonic-like stem cells (ELSCs) can be a new source of pluripotent stem cells which can play a promising role in regenerative medicine. In this study, SSCs were transdifferentiated into neuron-like cells (NLCs) using two-step differentiation protocol. pluripotency and germ cells markers were analyzed in SSCs and ELSCs. Also neural markers were analyzed in ELSCs and NLCs.

Methods:
Neonatal rat testes were mechanically dissected and digested then was cultured in DMEM supplemented with 15% FBS. The medium was replaced with DMEM containing LIF, mercaptoethanol, EGF, bFGF, and GDNF. After 5 weeks, ELSCs colonies appeared. SSCs and ELSCs were evaluated by Stra8, plzf (germ cells markers) Oct4, and sox2 (pluripotency markers) using qRT-PCR. The ELSCs colonies were isolated and cultured in DMEM containing 0.5 mM lithium chloride. In day 5, ELSCs transdifferentiated to NLC. They were evaluated using neural marker including Neurofilament 200 (NF-200), choline acetyltransferase (CAT), synaptophysin (Syp), Nestin (Nes), Neurogenin1 (NG1), Neurod1 (Nd1), and Neurofilament 68 (NF-68)gene expression.

Results:
Result showed increasing expression of Oct4 and sox2 genes and low level of Stra8 and plzf expression in ELSCs than SSCs. After neural transdifferentiation by lithium chloride induction, neural markers were examined by RT-PCR in ELSCs and NLCs. The result showed expression of NF-200, CAT, Syp, Nes, NG1, Nd1 and NF-68 in NLCs opposed to ELSCs.

Conclusion:
This study indicates lithium chloride can promote ELSCs to transdifferentiate into NLCs.

Keywords:lithium chloride, Embryonic-like stem cells, Spermatogonial stem cells, neural cells, Transdifferentiation

Introduction

Regenerative medicine is a new potential of using stem cells for treating the worst diseases millions of people are suffering from. There are three main stem cells sources : 1) embryonic stem cells, the cells of the inner cell mass of the blastocyst, 2)fetal stem cells which are derived from fetal tissues and form various organs of human body, 3) adult stem cells that exist in adult organs including brain, bone marrow, liver, and adipose tissue(1).

Lithium is used in psychopharmacotherapy as mood stabilizers for the treatment of bipolar disorder(2). Recent studies indicated neuroprotective effects of Lithium chloride (LiCl) in optic nerve and retina injury, ischemic stroke, spinal cord injury and axon regeneration in peripheral nerve trauma(3-8). These neuroprotective effects are mediated by inhibition of glycogen synthase kinase-3β (GSK-3β) activity. GSK-3β induces release of mitochondrial pro-apoptotic proteins(9, 10). Previous studies reported that lithium chloride could increase neuronal differentiation and proliferation of neural stem cells(11, 12).

SSCs are a subpopulation of type A spermatozoa and have an essential role for generation of spermatogonia for spermatogenesis and they can divide by maintaining their stemness as reserve stem cells (self-renewal)(13). In testis, SSCs are approximately 0.03% of the total germ cells(14). SSCs originate from primordial germ cells (PGCs). PGCs arise from extraembryonic mesoderm located in posterior part of primitive streak. In general, SSCs are capable of both spermatogenesis and self-renewal process(15).

Recently, studies have shown SSCs are pluripotent stem cells and they are able to differentiate into all cell lineages from three germ layers. SSCs can form ELSCs under germ cells culture condition without addition of pluripotency-associated genes or small molecules(16-18).kanatsu shinohara et al were the first researchers developed ELSCs in process of primary culturing of neonatal SSCs(19).more studies demonstrated the pluripotency of these cells by transforming them into germ line chimeras following injection into the blastocysts. The ELSCs not only show a morula-like structure similar to embryonic stem cells (ESCs) but also express markers identical to ESCs(16, 20-22). Other more studies have shown ELSCs can differentiate into various cell types under culture in growth factors and other differentiation conditions like hepatocytes, adipocytes , osteoblasts, cardiomyocytes , vascular smooth muscle cells, endothelial cells, and neurons(23-29). In this study SSCs was transdifferentiated into NLCs by a new simple method. In spite of Embryonic stem cells, SSCs as a novel Pluripotent stem cells doesn't have the ethical limitation. In addition, the allograft rejection is an important problem in research and clinical regenerative medicine. Immune response occurs after transplantation and patients need to use immunosuppressive drug for the rest of their life. Cleary, SSCs as a stem cell-derived tissue doesn't have immune rejection problems of transplantation(30)`.

Material and method

Generation and culture of SSCs

In this study testes were collected from five 4-day old neonatal rat (Sprague dawley) then testis tissue mechanically was decapsulated and dissected. For enzymatic digestion, testis tissue put in Dulbecco's modified Eagle's medium (DMEM) medium containing0.5 mg/ml collagenase/dispase, 0.5 mg/ml trypsin, and 0.05 mg/ml DNase with shaking and pipetting at 37°C for 30-45 minutes. after enzymatic digestion protocol, the mixture were cultured in DMEM (Invitrogen) supplemented with 15% FBS(31). In the following, these cells used for two-step differentiation process. In the first step, SSCs transitioned to ELSCs and in the next step ELSCs transdifferentiated to NLCs by lithium chloride induction.

ELSCs transformation

These cells including spermatogonia and Sertoli cells were transferred to DMEM medium containing 15% FBS, 1 mML glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM-mercaptoethanol (Invitrogen), 10^3 U/ml leukemia inhibitory factor (LIF)(Millipore), 20 ng/ml epidermal growth factor (EGF) (Invitrogen), 10 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen),and 10 ng/ml recombinant rat glial cell line-derived

neurotrophic factor (GDNF) (Invitrogen). In the first week, spermatogonial colonies were observed on top of the monolayer of Sertoli cells(17, 31). In third weeks, ELSCs colonies were appeared.

Gene Expression Analysis and Real Time RT-PCR

Real time RT-PCR was performed to analyze the expression of a subset of pluripotency markers as well as germ cell-specific genes. OCT4 and SOX2 as pluripotency genes and Stra8 and Plzf as spermatogonial markers were evaluated in ELSCs and SSCs.

Neural transdifferentiation for ELSCs

ELSCs colonies were collected from surface of plates by Pasteur pipette under the inverted light microscope and were isolated and transferred to DMEM medium containing 0.5 mg/ml trypsin and EDTA and put under shaking and pipetting. ELSCs were cultured in DMEM containing 15% FBS and 0.5 mM lithium chloride for five days. In day five, the morphology of ELSCs changed and NLCs appeared.

Gene Expression Analysis in ELSCs and NLCs

RT-PCR was performed to analyze the expression of neural marker genes such as Neurofilament 200, choline acetyltransferase, synaptophysin, Nestin, Neurogenin, Neurod1 and Neurofilament 68 in ELSCs and NLCs. The housekeeping gene, *Gapdh*, was selected for normalization of data in ELSCs and NLCs (32).

Result

ELSCs transformed from SSCs were used for neural transdifferentiation by lithium chloride induction. Two days after mechanical and enzymatic digestion of neonatal rat testis, testicular cells mixture started to attach on plates. In the first week, several colonies were formed on top of monolayer of Sertoli cells. At this stage, growth factors that needed for ELSCs transformation were added into medium. Medium was changed every 4 days and after 5 weeks ELSCs colonies appeared (Fig.1).

In the next step, Pluripotency and germ cells markers were examined by real time RT-PCR in SSCs and ELSCs. We selected stra8 and plzf as germ cells markers and sox2 and oct4 for pluripotency. The results demonstrated that the expression of sox2(12.17±4.73) and Oct4(3.35±0.04) were significantly increased in ELSCs ($P<0.01$). Also expression of star8 (51.92±9.27) and plzf (22.15±0.15) were significantly down regulated ($P<0.01$) (Fig.2). The results were expressed as mean ± standard deviation and were assessed by T-test (SPSS Software). P value less than 0.05 was considered statistically significant.

ELSCs colonies were isolated and transferred to DMEM medium containing trypsin and EDTA and put under shaking and pipetting. Then isolated cells transferred to new flasks (Fig.3A). These cells were cultured in DMEM containing lithium chloride. After 2 days, under induction of lithium chloride the morphology of ELSCs were started to change in neural appearance (Fig.3B). In day 5, all ELSCs transdifferentiated to NLCs (Fig.3C).

Neurofilament 200, choline acetyltransferase, synaptophysin, Nestin, Neurogenin, Neurod1, and Neurofilament 68 were selected as the neural marker genes for RT-PCR analyzing in ELSCs and NLCs. also, *Gapdh* was selected as a housekeeping gene for normalization of data. The results showed the expression of these genes in NLCs but not in ELSCs (Fig5).

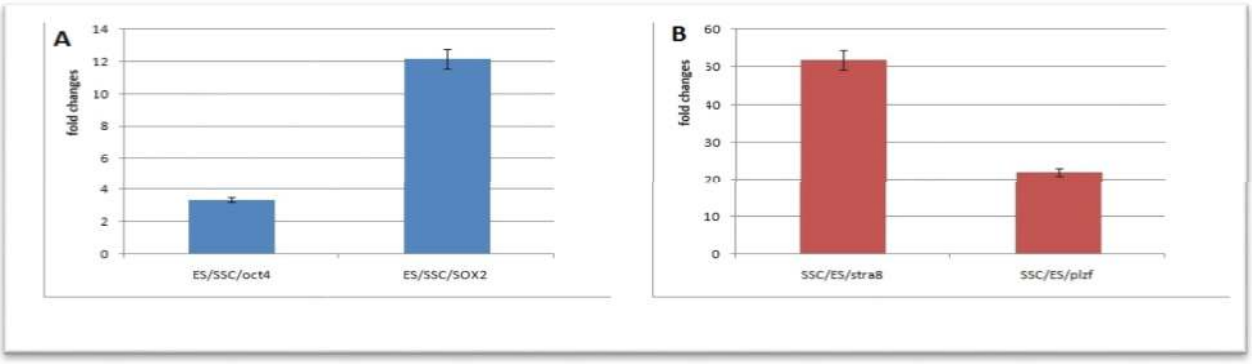

Figure 1. A) The culture of testicular cells mixture (Day 2) B) spermatogonial and Sertoli cells in DMEM with growth factors mentioned in protocol (week 1) C) ELSCs colony (week 5)

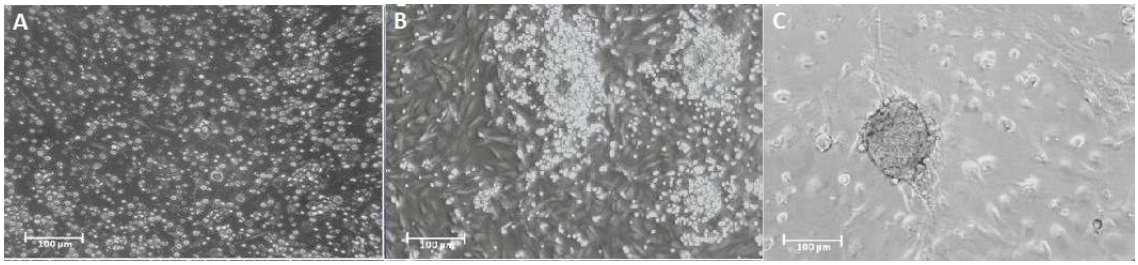

Figure2. A) Fold changes means showed high level of oct4 and sox2 expression in ELSCs in comparison with SSCs. B) Fold changes means showed low level of expression stra8 and plzf expression in ELSCs compared to SSCs

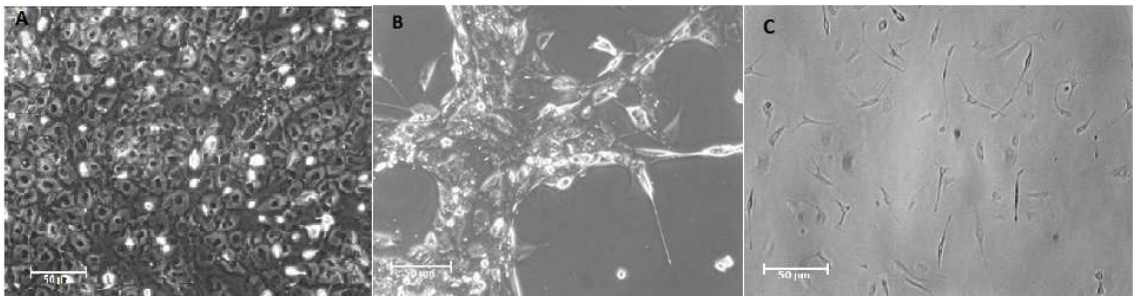

Figure3. A) Isolating and culture of Es like colonies B) ELSCs in neural transdifferentiation medium (after 2 days) C) NLCs after 5 days culture

genes	Primer sequences	TM	Product size
OCT4	TGTTCCTGTCACTGCTCTGG	59.61	164
	CCCCTGTTTGTGCTTTCAAT	56.79	
SOX2	TACCTCTTCCTCCCACTCCA	58.91	221
	CTCTCCCCTTCTCCAGTTCG	59.18	
Stra8	ACACCAGGCAACATCAACAA	58.23	169
	GGAACGGCAACAGGAAAATA	56.33	
Plzf	AAGGCAGCAGTGGACAGTTT	60.11	240
	CCCCTCTTTGCTCCTCTCTT	58.72	
Neurofilament 200	TTCCCTCCATGTCCACTCAC	59.02	240
	CCTTTTCTGGAGATGCAGCC	58.90	
choline acetyltransferase	TTCTGCTGTTATGGACCCGT	59.02	245
	AGATTGCTTGGCTTGGTTGG	59.03	
synaptophysin	GCATTCCTCAGCCCCTATCT	58.94	243
	AGCCTCCTCCACTCAGTCTA	59.00	
Nestin	GAGGTTCCCAGGCTTCTCTT	59.01	

	AGGACAGGGAGCCTCAGATA	59.06	245
Neurogenin	GCCCAAAGACCTCCTCCATA	58.79	195
	AGCAAGTGGTCAGGTATCCC	59.09	
NeuroD1	GGCACGTCAGTTTCACCATT	59.05	167
	CCCCTGTTTCTTCCAAAGGC	59.03	
Neurofilament 68	GTACCTGGAAGCAACCTCCT	59.02	217
	TCACCCTCACCACCTTCTTC	58.94	

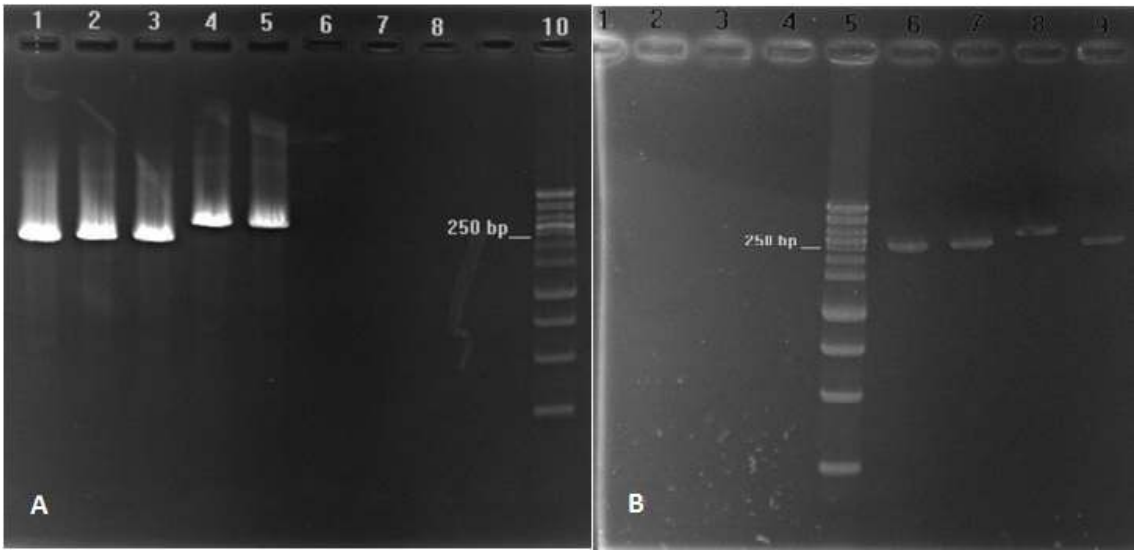

Figure4. The genes used for RT-PCR, the sequence of forward and reverse primers, the size of fragments, and melting temperature. (TM)

Figure5. Reverse transcription Polymerase Chain Reaction (RT-PCR) for Identification of neural marker in ELSCs and NLCs. A): 1) synaptophysin in NLCs, 2) NF_200 in NLCs, 3) choline acetyltransferase in NLCs, 4) GAPDH in NLCs, 5) GAPDH in ES like colonies, 6) synaptophysin in ES like colonies, 7) NF_200 in Es like colonies 8) choline acetyltransferase in Es like colonies, 9) empity, 10) ladder(50 bp). B): 1) Nestin in ES like colonies, 2) Neurogenin in ES like colonies,3) Neurod1 in ES like colonies , 4) Neurofilament 68 in ES like colonies, 5) Ladder(50 bp), 6) Nestin in NLCs, 7) Neurogenin in NLCs 8) Neurod1 in NLCs, 9) Neurofilament 68 in NLCs.

Discussion

Although we have not completely understood the mechanism of ELSCs reprogramming from SSCS, these cells can be used as a pluripotent stem cells source in research and clinic for regenerative medicine. Nervous system degenerative disorders are not only considered as irreversible problems but also have high degree of morbidity and mortality. However there is no definitive treatment for these disorders because of neurons necrosis, ELSCs can be presented as a promising stem-cell approach for neural regeneration due to their unique advantages and pluripotency(33). In our study we demonstrated that after transition of SSCs to ELSCs these pluripotent cells can be transdifferentiated to NLCs by lithium chloride induction (Two-step differentiation). In fact, lithium chloride could induce the expression of Neurofilament 200, choline acetyltransferase, synaptophysin, Nestin, Neurogenin, Neurod1, and Neurofilament 68 in these cells. There are some other studies along with our results(34, 35). These genes are expressed in different time spans during the nervous system development. Nestin as a intermediate filament protein is expressed in early stages of neural differentiation, then it is replaced with other neurofilaments to change the shape of cell(36, 37). Neurogenin and neuroD1 are basic helix–loop–helix transcription factors and are

expressed in the next stage. Neurogenin can change proliferative phase to neurogenesis phase(38). Studies reported that neurogenin-1 and neuroD1 are involved in neuronal differentiation(39). Neurofilament 68 and 200 are cytoskeletal proteins and play essential roles in axonal structure, caliber, and conduction velocity(40, 41). Synaptophysin as a presynaptic protein is expressed in the next stage and involved in endocytosis, docking, fusion, and membrane trafficking in differentiated neurons(42).

In 2006, ELSCs from SSCs were transdifferentiated to neurons and expressed neural transcription factors at the mRNA level and showed a neuron-like morphology. The mechanism of transition is still unknown though (33, 43). More studies were shown SSCs from neonatal piglet and chicken testes can be induced to differentiate into neuron-like cells (29, 44). Tin-Lap Lee et al used two-step protocol for generation of ELSCs from SSCs. SSCs were first dedifferentiated to embryonic stem-like cells then differentiated toward retinal lineages and transplanted into the retina of glaucoma mouse models by intravitreal injection. They demonstrated that the transplanted RGCs could survive in the host retina for at least 10 days after transplantation (45).

Li Qi et al studied the role of Lithium chloride in the proliferation and differentiation of neural stem cells (NSCs). They concluded that lithium chloride treatment could enhance the proliferation in NSCs and promote the dopaminergic neuronal differentiation of NSCs in vitro and in vivo. In Parkinson's disease models under lithium chloride treated NSCs, dendrite spine density increased and the learning and memory improved subsequently (46). In another study, the effect of lithium chloride on dopaminergic differentiation of human immortalized RenVm cells was investigated. RenVM is one of the immortalized human neural stem cell lines which is isolated from 10 week fetal neural ventral mesencephalon and was established with the V-myc oncogene by retroviral transduction. results indicated that Lithium chloride can be able to promote dopaminergic differentiation of RenVm cells in vitro(47). J Zhang et al shown Lithium chloride can promote the proliferation abilities of NSCs which is potentially mediated by activation of the Wnt signaling pathway. β-catenin expression increased and Gsk-3β expression inhibited significantly in NSCs treated with lithium chloride. increase in concentration of lithium chloride can lead to activation of NSCs in the G0/G1-phases, while NSCs were arrested in the S and G2/M-phases(48).also, lithium chloride can promote neural crest cells of hair follicles to differentiate into odontoblast-like cells by Wnt/β-catenin signaling(49). In addition, studies have shown lithium chloride play an essential role in nutrition, proliferation of stem cells, neural differentiation, and neuroprotection(50-53). Jin Seuk Kim et al in their study demonstrated Lithium increases neurogenesis and facilitates neural progenitor differentiation in dentate gyrus of hippocampus(54).

Conclusion

ELSCs from SSCs can be utilized for cell-based treatment in future medicine due to their advantages. Also, lithium chloride has been shown to promote neural transdifferentiation in these cells. Besides, in comparison to other neural differentiation techniques, lithium chloride is less complicated, more economical, and it's not that time consuming. Neurodegenerative disorders involve so many people around the world and new approaches can make a promising way towards it.

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