RESEARCH ARTICLE

Neurogenin-2 induced neuronal differentiation in co-cultured bone marrow stromal and neonatal cortical cells

Saba Majeed, Aisha Aziz, Kanwal Iftikhar, Shabana Usman Simjee

Abstract

Objective: To explore a novel and dynamic role for neurogenin-2 in promoting cortical neurogenesis in cells produced from co-culturing neonatal cortical neural progenitor cells with bone marrow stromal cells.

Method: The experimental study was conducted from June 2016 to January 2019 at the neuropharmacology laboratory of the Hussein Ebrahim Jamal Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, Karachi. The growth of cells at different stages in harvested cells was determined by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay. Immunocytochemistry was used to evaluate the protein expressions of neuronal markers and transcription factors. Data was analysed using SPSS 20.

Results: Data showed significant generation of neuronal cells and this was also verified by increased expression of nesting in cortical co-cultures with bone marrow stromal cells. Immunoreactive outcomes showed over expressions in co-cultured chlorotoxin cells. Subsequently, neurogenin-2 was found intermixed with induced expressions of transcriptional factor NeuroD1 and reduced glial fibrillary acidic protein-labelled cells.

Conclusion: Better understanding of the mechanisms underlying transcriptional modulation of neurogenic events hold the key for emerging treatment approaches towards neurodegeneration.

Key Words: Coculture, Glial Fibrillary, Bromides, Immunohistochemistry, Chlorotoxin, diphenyl, Neuropharmacology, Mesenchymal, Neurogenesis, Stem Cells.

(JPMA 74: S-68 (Supple-2); 2024) **DOI: https://doi.org/10.47391/JPMA-DUHS-S14**

Introduction

The development of appropriate in vitro strategies demonstrating differential potential of brain cells can minimise the gaps in understanding the functions of the nervous system. Progress in stem cell differentiation into neurons has opened the way for constructive strategies to understand the human brain better[1]. The in vitro co-culture system to generate neurons and promoting neurogenesis from neural progenitor cells (NPCs) may result in successful therapeutic approaches for neurodegenerative disorders along with cell replacement therapies. However, instant and effective procedures to produce neurons appears to be a problem with respect to the fabrication of a functional neuronal circuit. Moreover, the cure of neurological disorders also requires the production of specific neuronal lineages[2].

Pluripotent stem cells co-cultured with embryonic stem cells have been utilised to produce neuronal-like cells, or neurons. Their neuronal differentiation is a multifaceted process linked with several regulatory mechanisms and transcriptional factors[3]. It has been shown that co-culturing of isolated midbrain cells of neonatal mice with BMSCs substantially upregulated the neuronal expressing bone marrow stromal cells (BMSCs)[4].

To date, a wide range of transcription factors has been used in various protocols for induction of neuronal differentiation according to their contribution in developing brain and neurogenesis, assuming that they might play a role like stem cells. Although the resultant neurons are typically characterised once when they are differentiated, the role of regulatory cascades or transcriptional activation during process of differentiation are still unknown.[5] Further, studies have documented various important transcriptional regulators promoting neuronal differentiation in vivo and in vitro[6]. However, the precise and sufficient knowledge on fundamental factors mediating neurogenesis in differentiated stem cells has not been evaluated.

The recognition of possible transcriptional factors would enhance the differentiation processes of targetted neurons. A group of neural transcription family (basic helix-loop-helix [bHLH]), comprising Neurogenins (NGNs) and Neuro D, are major mediators of neurogenesis. The NGNs are key players for neuronal commitment and in vivo neuronal development.[7] The specified factors of this family have been utilised formerly to increase neuronal

H.E.J. Research Institute of Chemistry, International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan

Correspondence: Shabana Simjee. Email: shabana.simjee@iccs.edu

yield from differentiated mouse embryonic stem (ES) cells or cancer cells. Additionally, they have induced differentiated neuronal cells from human NPCs [8] and human fibroblasts.[9] Furthermore, the induction of NGN-2 in human stem cells and later the generation by glia cell co-cultures system has resulted in larger and homogenous yields of neuronal cells in only 14 days.[10] Previously, both NGNs independently induced neuronal lineages from stem cells and their co-expressions were found in NPCs in vivo.[11] Moreover, the fate specification and activation of pronounal genes is linked with NGN-2. The regulation of neural fate by NGN-2 is committed with reduced expressions of glial genes in neural progeny. NGN-2 also augments proliferated state of progenitor cell, hence preserving the undifferentiated cells before conversion to granule cells by NeuroD1 activation.[12]

To the best of our knowledge, there is no appropriate data yet that represents how upregulated NGN expressions efficiently modulate neurogenic differentiation. In addition, the role of NGN-2 in BMSCs differentiation or co-cultured BMSCs with cortical neurons is not elucidated and co-culturing strategy may advance the therapeutic objectives of transplantation therapy and neurodegenerative disorders. The current study was planned to fill the gap in literature by exploring a novel and dynamic role for NGN-2 in promoting cortical neurogenesis in cells produced from co-culturing neonatal cortical NPCs with BMSCs.

Materials and Methods

The experimental study was conducted from June 2016 to January 2019 at the neuropharmacology laboratory of the Husein Ebrahim Jamal (HEJ) Research Institute of Chemistry, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi. The wistar rat (120-150gm) and their two days old pups were raised at the ICCBS animal house. The experiments were conducted under ethical principles designed by the National Institutes of Health (NIH) for use of animals for experimental purposes. Approval was obtained from the institutional advisory committee on animal standards.

After flushing the bone marrow from the shaft of the femur bone, it was centrifuged and the resultant cell pellet was collected, and 1mL of Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% FBS (foetal bovine serum)along with 1% pen strep and sodium pyruvate was added. Before seeding the cells, the number of viable cell was determined by trypan blue exclusion method[13]. The cells were distributed into 2 equivalent portions. the first portion was grown as a control group and the other one was co-cultured with dissociated cortical cells freshly dissected from neonatal rat pups. The concentration of cells for co-cultures was set at 160,000 cells/mL that contained 1:2 ratios for cortical and BMSCs, respectively. The cultured flasks were then incubated at 37ºC and 5% carbon dioxide (CO_2) for 24 hours. After that, the floating haematopoietic cells and debris were removed and the adherent cells were further kept in incubation and monitored properly until the cells got approximately 80% confluent.

DMEM was then aspired and the cells were splashed with 1X phosphate buffer saline (PBS) twice. To obtain cell pallet, 1mL trypsin- ethylenediaminetetraacetic acid (EDTA) (0.05%) solution was used. Cell cultures were incubated at 37°C for 4min for the detachment of cells from the flask. The action of trypsin-EDTA was deactivated by adding 5% DMEM. The pellet was obtained by centrifugation of cell suspension at the speed of 1000 rpm for 8min. The resultant pellet was re-suspended in 1mL fresh 5% DMEM, and evenly divided into 2 cell culture flasks to grow. Both flasks were labelled with passage 1 (P1), and incubated at former conditions in the incubator.

The viability of full-grown cells at different time periods was assessed by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. The cells were trypsinised and seeded onto 96-well plate at the density of 8000 cell/well, and kept for 24h incubation at 37ºC. The next day, MTT dye (0.5 mg/mL; Sigma-Aldrich, CAS-No, 298931) was added to each and every well after removal of culture medium, and the plates were re-incubated for 3h. After incubation, the dye was aspirated and 100µL of DMSO (Dimethyl sulphoxide) was added. Percentage of viable cell was calculated by the formation of purple formazan crystals. The absorbance was measured using a spectrophotometer (TECAN Trading AG, Switzerland) at the wavelength of 570nm. The recorded absorbance revealed the relative number of proliferating cells in different cultured groups. All assays were conducted in triplicates.

For immunocytochemical analysis, the confluent cells were dispensed in chambered slides. The cells were subjected to fixation with treatment of 4% paraformaldehyde. Subsequently, the cells were cleaned with PBS at least three times and incubated for 1h in the blocking solution, which comprised 2% bovine serum albumin (BSA), 2% normal goat serum and 0.2% Tween20, at 37ºC. After 1h incubation, the cells were splashed thrice with PBS. The dilution of each primary antibody (glial fibrillary acidic protein [GFAP], nestin, NeuroD1 and NGN-2) was prepared in PBS with concentration ranging 8-

15µg/ml as per the protocol (R and D). The slides were then kept for overnight incubation at 4°C. Following incubation, washing was done three times with PBS, and the secondary antibody (1:200) was added and re-incubated at room temperature for 60min. The cells were washed thrice and stained with DAPI (1:500). Stained wells were mounted with PBS/glycerol (1:1) and examined under 20X magnification with a fluorescent microscope (Nikon, Japan) equipped with Nuance 2.0 software.

The immunofluorescent photographs were evaluated using ImageJ software. The intensity of fluorescence was calculated from randomly selected fields after subtracting the background intensity. Percentage intensity of protein expression was calculated as per the counting of cells/field. The data was composed of 3 individual experiments.

Data was analysed using SPSS 20. Data was expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to assess the difference between various groups. P<0.05 were considered statistically significant.

Results

The morphology of BMSCs after 72h of seeding appeared as tiny spheres with adherent potential. However, 2 weeks later, the bulk of cells displayed large spindle-like irregular cells showing long processes (Figure 1). After 14-28 days, the cells were 80% confluent and transformed into homogenous clusters, like fibroblast cells grown in parallel way. The cells were kept incubated till P1 and P3 passages of cells.

The co-culturing of BMSCs and cortical cells was done at a ratio of 1:2, respectively. After 24 h, a mixture of adherent cells was observed under the microscope. Around 7-14 days after incubation, most cells expressed minute processes with irregular shape. Morphological variations from BMSCs were analysed comparable to co-cultured BMSCs with cortical cells (Figure 1). A large population of co-cultured cells showed resemblance to cortical cells following 2 weeks. Fibroblastic structures were rarely observed. However, significant growth of cells showed particularly differentiated and specialised morphology identical to cortical cells. After day 14, neuronal connections having retraction in the cell body were observed. The homogenous neuronal cells maintained their morphology till P2 and P3.

The percentage of viable cells was estimated with MTT assay on days 3, 7 and 14 after culturing. The maximum no of live cells was assessed in co-cultured group, whereas

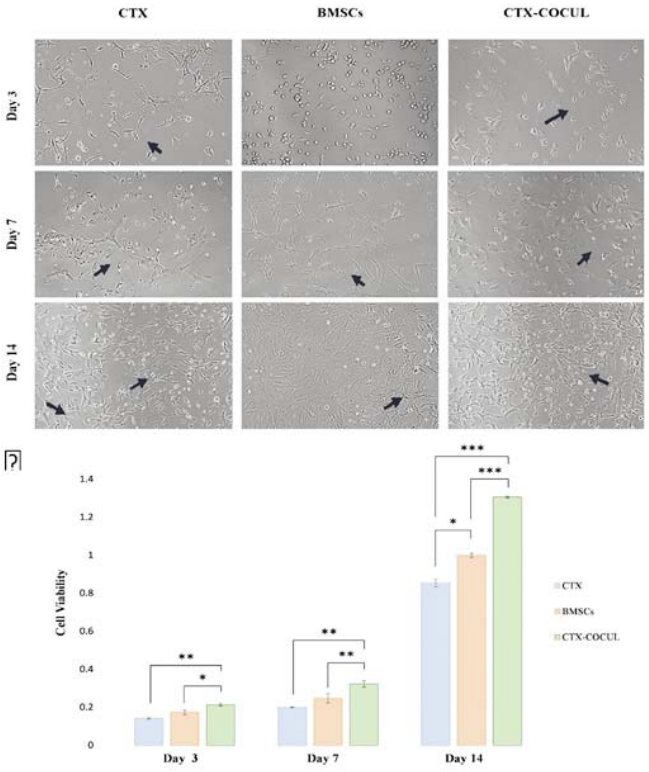

Figure-1: Morphological variations and increased proliferation of co-cultured cells. bone marrow stromal cells (BMSCs) appeared as fibroblast cells growing in parallel way. (I) A large population of co-cultured chlorotoxin (CTX-COCUL) cells were identical to cortical cells following 14 days. (II) Statistically significant difference was demonstrated among CTX-COCUL cells and BMSCs (***p<0.001) on days 7 and 14. Arrow keys show distinct synaptic alteration in proliferated cells between cortical and CTX-COCUL cells (###p<0.001).

the BMCSs and cortical cells showed significantly reduced viability compared to the co-cultured cortical cells. Significant difference was noted among co-cultured chlorotoxin CTX-COCUL) cells and BMSCs (p< 0.001) on days 7 and 14 (Figure 1). Distinct alteration in proliferated cells between cortical and CTX-COCUL cells was noted (p<0.001).

Neurogenic differentiation of co-cultured cortical NPCs and BMSCs was evaluated through immunoreactivity for neural and glial progenitor markers. Furthermore, immature neuronal expressions were examined with tubulin fluorescence levels. The results of immunocytochemistry exposed the significant upregulation of nestin and GFAP in the cortical cells compared to BMSCs where the expression of GFAP was absent and minor expressions of nestin were examined (Figure 2). In case of co-cultured cells, significantly higher immunoreactivity of nestin was expressed, while the glial marker was down-regulated compared to the cortical cells (Figure 2). In CTX-COCUL cells, the comparable

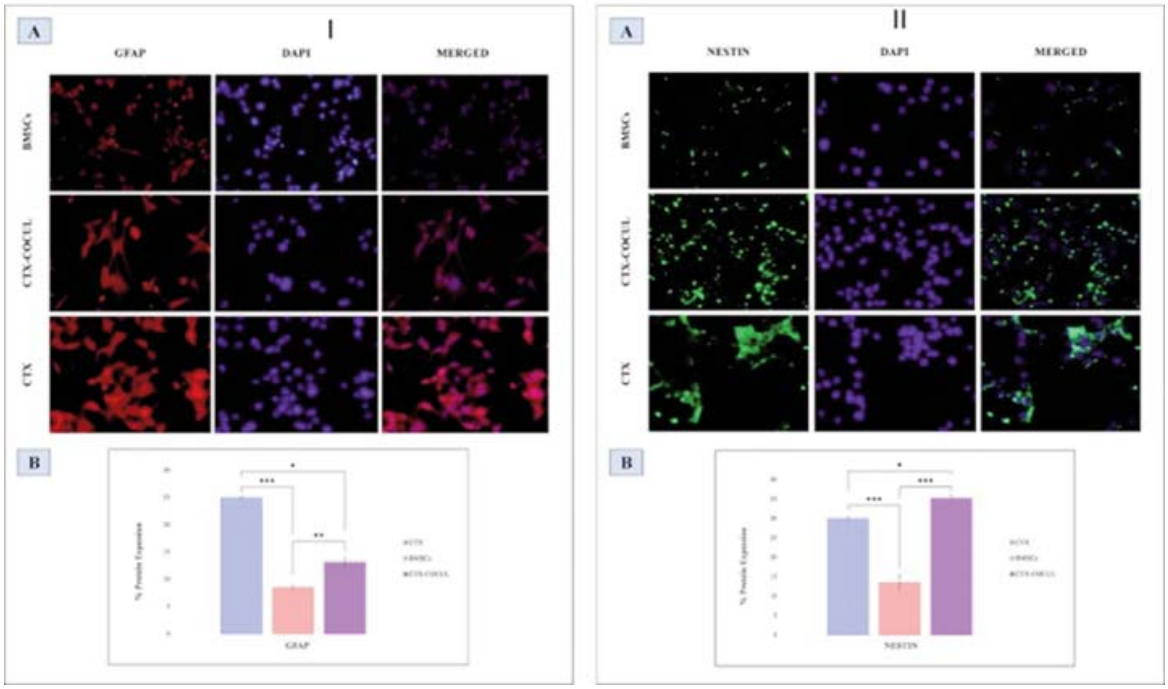

Figure-2: Potential increase in protein expression of glial fibrillary acidic protein (GFAP) and nestin, which were determined through immunocytochemistry of co-cultured chlorotoxin (CTX-COCUL) cortical cell. In case of co-cultured cells significant higher expression of nestin was found, while the glial marker was down=regulated compared to the cortical cells (IA and IIA). Expression levels were significantly increased in cortical cells compared to the bone marrow stromal cells (BMSCs). In CTX-COCUL cells, the comparable morphology to neuronal-like cells was sustained. Data was expressed as mean ± standard error of mean (SEM) (n=3). ** and *** show significant results compared to BMSCs and CTX cells (IB and IIB, respectively).

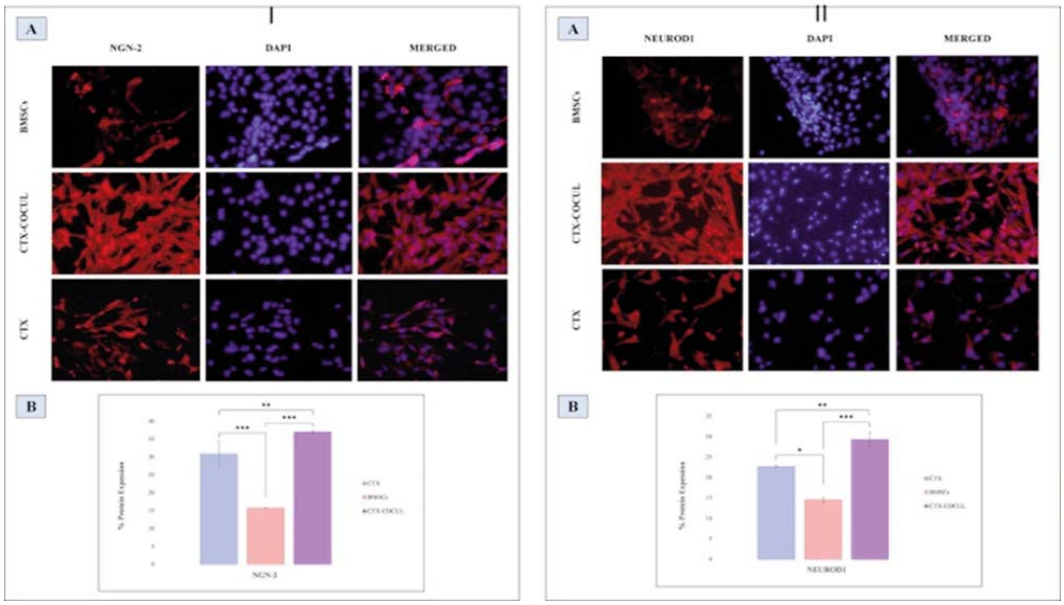

Figure-3: Neurogenin-2-mediated enhanced neuronal differentiation in cells. Significant NGN expressions were noticed in cortical cells and co-cultured chlorotoxin (CTX-COCUL) cells (IA-B). Enhanced NeuroD1 expression was found in CTX-COCUL cells compared to BMSCs, and cortical cells alone (IIA-B). Elevated NeuroD1 expression demonstrated increased neurogenesis. Data was expressed as mean ±standard error of mean (SEM) (n=3). ** and *** showed significant results compared to bone marrow stromal cells (BMSCs) and CTX cells, respectively.

morphology to neuronal-like cells as well as the immunophenotypic alteration towards neuronal fate was sustained till 2 passages, and it was achieved without the addition of N2/B27 (ndiff neuro-2 Medium supplement) or EGF (epidermal growth factor) and any conditioning strategy, like treatment with neurobasal medium.

Significant NGN-2 (Figure 3) expressions were detected in cortical cells and CTX-COCUL cells. However, comparative measurements showed significant over expressions in co-cultured cells, thus representing the role of NGN-2-induced neuronal differentiation of co-cultured cells. The results were also examined by comparison with expression of NGN-2 in BMSCs (Figure 3). The NGN-2expressions were hardly observed compared to CTX-COCUL and cortical cells. This demonstrated high immune expressions of NGN-2 in co-cultured cells due to fate alteration from mesenchymal to neuronal lineage which might be regulated with co-culturing method. The outcomes also proved that NGN-2 regulated the neuronal induction in BMSCs, or the cells originated from co-cultures.

Immunoreactivity was checked for NeuroD1 to confirm the onset of differentiation and cortical neurogenesis in BMSC-derived CTX-COCUL cells. Significant increase in the NeuroD1 expression was found in CTX-COCUL cells compared to BMSCs and cortical cells alone (Figure 3). The marked expression of NeuroD1 correlated with the over-expression of NGN in co-cultures that demonstrated that the increase in neurogenesis or differentiated neurons were the results of transcriptionally-induced NGN along with NeuroD1.

Discussion

The current study examined the expression of NGN-2 by using the co-culturing approach. As transcription factor, bHLH family of neurogenins transitorily expressed pro-neural protein in DG progenitors before their differentiation into granule neurons [13]. Furthermore, it is vital for proper growth of the DG neurons[11,14]. However, its expression in case of cortical neurogenesis and co-cultured-derived cortical neurons has not been studied previously.

The present study demonstrated increased NGN-2 immunoreactivity in cortical and co-cultured cortical neurons. However, the expression in case of CTX-COCUL more compared to the cortical-only cells. Concurrently, the high expressions of nestin displayed direct correlation with the upregulation of NGN-2. These outcomes supported the evidence that the proliferation of neural progenitors was related to the upregulated expressions and activation of NGN-2 since pronounal bHLH proteins regulate the production of progenitor cells and their differentiation towards neurogenic programme of the nervous system[15]. Moreover, NGN-2 is importantly linked to limit differentiation of the multipotent progenitors into the astrocytic cells, and tend to direct the progenitor's fate towards neural specification[16]. The GFAP immunoreactivity in co-cultures exhibited reduced levels compared to grown cortical cultures. This linked with the fact that NGN-2 constrained differentiation into glial progeny and excited cell differentiation towards neuronal fate. The less expression of GFAP compared to NGN-2 and nestin in CTX-COCUL confirmed that co-culturing of CTX cells with BMSCs had the capacity to boost neural progenitor differentiation into particular neuronal cortical cells with increased expressions of NGN-2.

Interestingly, in addition to the cortical cultures, a marked upregulation in calretinin expression was also detected in CTX-COCUL. These findings proposed an approach of stem cell differentiation, especially BMSCs, into neurons through significant increments of NGN-2 intensities. To further confirm this approach, the current study also explored another transcription factor NeuroD1 related to NGN-mediated neurogenesis in cortical or co-cultured cells. The stimulation of NeuroD1 is evident to instigate the neurogenesis[17,18], and NGN-2 is associated with conserving the undifferentiated state of progenitor cell by augmenting NPC proliferation before differentiation into granule cells regulated by expression of NeuroD1 genes[19]. Guo et al. revealed reactive microglial conversion by retroviral expression of NeuroD in Alzheimer's disease and aged mice models. However, inducing expressions of NeuroD1/NGN-2 to control neurogenesis for the treatment of neurodegenerative illness is still challenging.[20]

NeuroD1 and NGN have induced in vitro differentiation in neuroblastoma.[21] On the contrary, the current results expressed prominent NeuroD1 expression in cortical cultures compared to BMSCs, but at the same time CTX-COCUL revealed significant over-expressions compared to cortical cell culture. All these findings are in accordance with previous studies and were indicative of enhanced proliferation with increased expression of neuronal markers.[22] The in vitro evaluation of co-culturing the cells by inducing transcriptional activation of NeuroD1 and NGN-2 has never been studied formerly. However, the current observation demonstrates the significant expression of NGN-2 compared to CTX cells, which validates the result of amplified levels of both factors in CTX-COCUL cells compared to cortical cells, and confirms the initiation of neuronal sequencing in co-cultured cells.[23]

The current study has limitations as the findings presented relate to research that was completed 5 years ago. The delay in publication was owing to inadvertent circumstances, and may have affected the worth of the findings. It was owing to the novelty of the research related to mechanisms regarding neuronal transcription factors that the results have been made public. However, advanced working is in progress and the results would be published immediately.

Conclusion

Upregulated transcriptional factors NGN-2 and NeuroD1, and neural protein expressions might be the result of the onset of neurogenesis in CTX-COCUL cells. These findings provide an important insight into molecular mechanisms and transcriptional modulation of neurogenic events for emerging treatment approaches towards neurological diseases.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: None.

References

1. Amamoto R, Arlotta P. Development-inspired reprogramming of the mammalian central nervous system. Science 2014;343:1239882. doi: 10.1126/science.1239882.
2. Barker RA. Stem cells and neurodegenerative diseases: where is it all going? Regen Med 2012;7:26-31. doi: 10.2217/rme.12.64.
3. Zakrzewski W, Dobrzyński M, Szymonowicz M, Rybak Z. Stem cells: past, present, and future. Stem Cell Res Ther 2019;10:68. doi: 10.1186/s13287-019-1165-5.
4. Qi X, Shao M, Peng H, Bi Z, Su Z, Li H. In vitro differentiation of bone marrow stromal cells into neurons and glial cells and differential protein expression in a two-compartment bone marrow stromal cell/neuron co-culture system. J Clin Neurosci 2010;17:908-13. doi: 10.1016/j.jocn.2009.10.013.
5. Guo N, McDermott KD, Shih YT, Zanga H, Ghosh D, Herber C, et al. Transcriptional regulation of neural stem cell expansion in the adult hippocampus. Elife 2022;11:e72195. doi: 10.7554/eLife.72195.
6. Busskamp V, Lewis NE, Guye P, Ng AH, Shipman SL, Byrne SM, et al. Rapid neurogenesis through transcriptional activation in human stem cells. Mol Syst Biol 2014;10:760. doi: 10.15252/msb.20145508.
7. Dennis DJ, Han S, Schuurmans C. bHLH transcription factors in neural development, disease, and reprogramming. Brain Res 2019;1705:48-65. doi: 10.1016/j.brainres.2018.03.013.
8. Le Dréau G, Escalona R, Fueyo R, Herrera A, Martínez JD, Usieto S, et al. E proteins sharpen neurogenesis by modulating proneural bHLH transcription factors' activity in an E-box-dependent manner. Elife 2018;7:e37267. doi: 10.7554/eLife.37267.
9. Ladewig J, Mertens J, Kesavan J, Doerr J, Poppe D, Glaue F, et al. Small molecules enable highly efficient neuronal conversion of human fibroblasts. Nat Methods 2012;9:575-8. doi: 10.1038/nmeth.1972.
10. Zhang Y, Pak C, Han Y, Ahlenius H, Zhang Z, Chanda S, et al. Rapid single-step induction of functional neurons from human pluripotent stem cells. Neuron 2013;78:785-98. doi: 10.1016/j.neuron.2013.05.029.
11. Hulme AJ, Maksour S, St-Clair Glover M, Miellet S, Dottori M. Making neurons, made easy: The use of Neurogenin-2 in neuronal differentiation. Stem Cell Reports 2022;17:14-3. doi: 10.1016/j.stemcr.2021.11.015.
12. Lin HC, He Z, Ebert S, Schörnig M, Santel M, Nikolova MT, et al. NGN2 induces diverse neuron types from human pluripotency. Stem Cell Reports 2021;16:2118-27. doi: 10.1016/j.stemcr.2021.07.006.
13. Avelar-Freitas BA, Almeida VG, Pinto MC, Mourão FA, Massensini AR, Martins-Filho OA, et al. Trypan blue exclusion assay by flow cytometry. Braz J Med Biol Res 2014;47:307-15. doi: 10.1590/1414-431X20143437.
14. Nieto M, Schuurmans C, Britz O, Guillemot F. Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. Neuron 2001;29:401-13. doi: 10.1016/s0896-6273(01)00214-8.
15. Liu W, Zhou H, Liu L, Zhao C, Deng Y, Chen L, et al. Disruption of neurogenesis and cortical development in transgenic mice misexpressing Olig2, a gene in the Down syndrome critical region. Neurobiol Dis 2015;77:106-1. doi: 10.1016/j.nbd.2015.02.021.
16. Wen S, Li H, Liu J. Dynamic signaling for neural stem cell fate determination. Cell Adh Migr 2009;3:107-1. doi: 10.4161/cam.3.1.7602.
17. Pataskar A, Jung J, Smialowski P, Noack F, Calegari F, Straub T, et al. NeuroD1 reprograms chromatin and transcription factor landscapes to induce the neuronal program. EMBO J 2016;35:24-45. doi: 10.15252/embj.201591206.
18. Roybon L, Deierborg T, Brundin P, Li JY. Involvement of Ngn2, Tbr and NeuroD proteins during postnatal olfactory bulb neurogenesis. Eur J Neurosci 2009;29:232-43. doi: 10.1111/j.1460-9568.2008.06595.x.
19. Beckervordersandforth R, Zhang CL, Lie DC. Transcription-Factor-Dependent Control of Adult Hippocampal Neurogenesis. Cold Spring Harb Perspect Biol 2015;7:a018879. doi: 10.1101/cshperspect.a018879.
20. Guo Z, Zhang L, Wu Z, Chen Y, Wang F, Chen G. In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. Cell Stem Cell 2014;14:188-202. doi: 10.1016/j.stem.2013.12.001.
21. Kim HJ. Stem cell potential in Parkinson's disease and molecular factors for the generation of dopamine neurons. Biochim Biophys Acta 2011;1812:1-11. doi: 10.1016/j.bbadis.2010.08.006.
22. Majeed S, Aziz A, Simjee SU. Neuronal transcription program induced in hippocampal cells cocultured with bone marrow derived mesenchymal cells. Heliyon 2020;6:e05083. doi: 10.1016/j.heliyon.2020.e05083.
23. Niklison-Chirou MV, Agostini M, Amelio I, Melino G. Regulation of Adult Neurogenesis in Mammalian Brain. Int J Mol Sci 2020;21:4869. doi: 10.3390/ijms21144869.