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Research article

Neuronal transcription program induced in hippocampal cells cocultured with bone marrow derived mesenchymal cells

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ABSTRACT

Several approaches have been applied to harvest bone marrow stromal cells (BMSCs) and to differentiate into neurons or neuronal-like cells through chemical stimulation or exposing to growth factors. To date, the data regarding induction or regulation of neuronal transcription program in neuronal-like cells derived from BMSCs is yet unknown. The objective of this study is to co-culture BMSCs with neonatal hippocampal cells and generate neuronal-like cells by direct cell-to-cell contact system without using neuronal growth factors or neurobasal medium. Here, we proposed a role for NeuroD1 and Neurogenin -2 bHLH family of transcription factors implicated in onset of neurogenesis and differentiation of cells into neurons in promoting the interaction of hippocampal cells with BMSCs and their differentiation in to neurons. The proliferation of the cells was assessed with MTT assay and the role of neuronal induction and differentiation transcription regulators NeuroD1 and Neurogenin-2 in cocultured cells was determined through immunocytochemical analysis. We observed activation and expression of the neurogenic transcription factors NeuroD1 and NGN-2 associated with neuronal activation program to initiate the onset of neurogenesis in cocultured cells. Further, our results have shown a significant expression of neuronal progenitor and immature neuronal marker i.e., nestin and tubulin respectively in cocultured cells endorsing the initiation of neuronal activation.

1. Introduction

In many organs, self-renewal is supported by stem cells that differentiate into a narrow cell type related to the organ in which they are harboured [1, 2, 3, 4, 5, 6]. In bone marrow, the multipotent stem cells fulfils the demand of diverse cells like monocytes, lymphocytes, granulocytes, platelets and RBCs [7]. The non-hematopoietic multipotent cells are usually called bone marrow stromal cells (BMSCs) or mesenchymal stromal cells (MSCs) having differentiating ability of mesenchymal source or cells produce from the support structures of the bone marrow [7, 8]. BMSCs have been reported for their differentiating capability into hepatocytes, skeletal and cardiac muscle [9, 10, 11, 12], as well as, glial- and neural-like cells [13, 14]. The formation of neuroectodermal tissue from BMSCs is of particular interest. *In vitro* studies have revealed that BMSCs, in the presence of certain growth factors have the capacity to differentiate into neuroectodermal-like cells. An adult mammalian brain has limited regenerative capacity following injury in specific regions [15]. Even though it is now possible to produce cultures of neural stem cells (NSCs) from adult brain tissues [16, 17], it is still difficult to separate or isolate such cells. Current studies related to transplantation of BMSCs into the developing mouse brain have shown to produce astrocytic cells in a limited number [18, 19]. Studies have reported that undifferentiated BMSCs transplantation in rats demonstrates therapeutic advantage after ischemic brain injury [20], traumatic brain injury [21, 22], or spinal cord injury [23]. However, the difficult access of NSCs deep in the brain severely limits clinical efficacy. A recent report indicating that NSCs produce hematopoietic cells *in vivo* propose that populations of stem cells may be less restricted than was previously supposed [24]. It has been evident that in neonatal mice, the introduction of MSCs into the lateral ventricles can enhance differentiation of MSCs into neurofilament-containing cells and astrocytic cells provide support to this contention [19]. One of the study briefly describes the differentiation of rat and human MSCs into neurons, and the potential therapeutic advantages of this approach in the treatment of neurological diseases [14].

It has been shown that coculturing of fetal mouse midbrain with BMSCs significantly enhances the percentage of glial fibrillary acidic protein (GFAP) and NeuN (marker of astroglia and neurons) expressing

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BMSCs. The experiments of co-culture support the hypothesis that signaling with trophic factors and cytokines in addition to cell–cell contact, plays a vital role in differentiation of these BMSCs.

NeuroD1 is a transcription factor belonging to the family of basic helixloop-helix protein. It serves as an indicator of neurogenic differentiation for neurogenesis, which may presented as a gene for neuronal determination. It is required for the survival of adult born neurons [25]. Another transcription facto Neurogenin 2 (Ngn2) belonging to a bHLH is linked with both neural specification and neurogenesis. Ngn2 transcription factor increases expression of proneural genes and constrain neural fate by inhibiting glial genes expression in NPCs. Ngn2 is associated with progenitor cell proliferation before NeuroD1 expression hence maintaining the undifferentiated state before commitment to granule cells by NeuroD1 expression [26, 27]. Ngn2 is associated with progenitor cell proliferation before NeuroD1 expression hence maintaining the undifferentiated state before commitment to granule cells by NeuroD1 expression there is no sufficient data that how NeuroD1 and Neurogenin upregulated efficiently. NeuroD1 and neurogenin can induce differentiation in neuroblastoma *in vitro* [28, 29]. On the contrary, NeuroD1 is found to be increased in expression and promoting neuroblastoma [30]. However its role in differentiation of BMSCs or cocultured BMSCs with neurons is not identified and can be set as innovative target in neurodegenerative disorders and transplantation therapy.

2. Material and methods

2.1. Isolation of bone marrow stromal cells and co-culturing with hippocampal cells

Wistar rats (150–200 gm) were used to isolate bone marrow (BM) from their femurs. All animals were housed in animal housing facility of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. Experiments were performed in accordance to the guidelines of NIH set for the care and use of animals for experimental procedures and after protocol were approved with the issuance of protocol number 0012–2018, assigned by Advisory Committee on Animal Standards, ICCBS, University of Karachi. The isolated BM was added into Dulbecco's Modified Eagle's medium (DMEM) supplemented with 5% FBS, 1% penicillin/streptomycin solution, 1% sodium pyruvate and L-glutamine. The BM was then centrifuged at 180 g for 8 min. Following centrifugation, cell pellet was collected and 1 ml of fresh DMEM was added. The cell viability was checked using trypan blue exclusion method [31]. The cells were then divided into two equal parts. One part was cultured as a control and the second part was co-cultured with freshly isolated hippocampal cells in T75 cm^2 flask. The hippocampal cells were isolated from the 2 days old rat pups under sterile condition. The cell density for co-cultures was set at 140,000 cells/ml containing 1:2 ratios of BMSCs and hippocampal cells respectively. The same cell density was set for only HP and BMSCs groups. The cultured flasks were then incubated at 37 °C and 5% CO_2 for 24 h. Next day, the non-adherent hematopoietic cells and dead debris/tissues of hippocampus were removed and the adherent cells were further incubated and checked regularly till the cells attain 80–90% confluency.

2.2. Morphology of the primary cultured neurons and cocultured (COCUL) cells

To evaluate the morphological difference among cocultured, hippocampal cells and BMSCs, the cultured cells from all groups were examined at various time points i.e., on day 3, 7 and day 14 after seeding into 6-well plates. For morphological variation between all groups, images of the cultured cells were captured at aforementioned time period using Nikon TE-2000 inverted microscope equipped with phase-contrast optics.

2.3. Cell proliferation analysis

Cell proliferation studies were performed with MTT 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). Briefly, the cultured cells at various time periods were dispensed into each well of 96-wells plate and incubated to adhere for 24 h at 37 °C. Following incubation, media was aspirated and MTT dye was added to each well. Following 3 h incubation supernatant was removed and 100 μL of DMSO was added into each well. Percent cell viability was estimated by conversion of the yellow thiazolyl blue tetrazolium bromide (0.5 mg/ml; Sigma-Aldrich) to the purple formazan [32]. The plates were incubated on an orbital shaker for 10–20 min until formazan crystals were solubilize. The absorbance was recorded at 560 nm using spectrophotometer (TECAN Trading AG, Switzerland). The assays were executed in triplicates. The corrected absorbance was calculated by subtracting the blank reading of culture medium from assay absorbance recorded with cultured cells. The measured absorbance was proportional to number of proliferated/viable cells [32].

2.4. Immunocytochemical analysis

Cells were seeded in 2-chambered slides (Lab-Tek®) and once confluent, they were treated with 4% paraformaldehyde fixation process. The cells were washed thrice with PBS followed by incubation in blocking solution (2% bovine serum albumin (BSA), 2% normal goat serum and 0.2% Tween20, all prepared in PBS) at 37 °C for 1 h. After incubation, all cells were washed 5x with PBS. Primary antibody in dilution of 1:100 was added and cells were further incubated overnight at 4 °C. Next day, the cells were washed 3X with PBS secondary antibody (1:200 dilutions in PBS) was added and reincubated for 1 h at room temperature. The cells were washed and stained with 4, 6-diamidino-2-phenylindole (DAPI) in 1:500 dilutions in PBS. The DAPI staining was performed to analyze nuclei. Mounting of the stained slides was done with PBS/glycerol (1:1) and viewed under a fluorescent microscope (Nikon, Japan). The antibodies used for immunocytochemical analysis are shown in Table 1.

2.5. Image analysis

The images were analyzed using ImageJ software. For quantification studies, 5 fields were selected blind based and the intensity of fluorescence was calculated. The background intensity of each image was subtracted from it so as to confirm the expression of aforementioned proteins in the remaining particles. Percentage intensity of protein expression was calculated as per counting of cells/field. Data from three different experiments were combined and expressed as means ± SEM. All images were captured at 20x magnification.

2.6. Statistical analysis

The data are expressed as the mean ± SEM for graphical representation. The data analysis was performed using Statistical Package for Social Sciences (SPSS v20). One-Way Analysis of Variance (ANOVA) test was used to analyze the statistical data for significance. The analysis was done by comparison between different groups. The significant results presenting P value <0.05 were labeled as *, <0.01 as ** and <0.001 as ***.

3. Results

3.1. Morphological assessment of BMSCs, hippocampal cells and cocultured cells

The BMSCs cultured for 3 days were seen as small sphere adherent cells. However, between 7-14 days, the majority of cells exhibited

Table 1. Primary and secondary antibodies used for immunochemical staining.

Markers	Primary Antibodies	Dilution of Primary Antibodies	Secondary Antibodies
NESTIN	Monoclonal mouse IgG2A MAB2736	8–25 μg/ml	Alexa Flour ®^{488} Goat anti-rabbit IgG (H + L)
NEUROD1	polyclonal goat IgG AF2746	5–15 μg/ml	Alexa Flour ®^{546} Donkey anti-goat IgG (H + L)
NEUROGENIN	Mouse monoclonal IgG MAB3314	8–25 μg/ml	Alexa Flour ®^{546} Goat anti-mouse IgG (H + L)
TUBULIN(anti Tuj-1)	Monoclonal mouse IgG2A MAB 1195	8–25 μg/ml	Alexa Flour®^{546} Goat anti-mouse IgG (H + L)

irregular large spindle shaped morphology with extended processes (Figure 1A). After 14 days, the cells were not only fully confluent but their morphology gradually turns into fibroblastic-like structures with distinct homogenous colonies growing in parallel manner. The cells were subjected to proliferate until P1 and P3.

For primary co-culture of hippocampal cells and BMSCs, the cells were seeded at a ratio of 1:2 respectively followed by incubation in DMEM (low FBS 5%) without any neural inducing chemical or growth factor. A large number of mixed adherent cells were observed after 24 h of co-culture. Between 1-2 weeks after incubation, 80% cells demonstrated irregular morphology with tiny processes. Distinct morphological changes from BMSCs were observed in comparison to cocultured BMSCs with hippocampal cells (Figure 1A&B). Most of the co-cultured cells resemble to hippocampal cells at day 14. Some growth of fibroblast like cells was also observed. However huge number of confluent cells showed specific differentiated morphology like hippocampal neurons. After day 14 extended processes with retracted cell body was also observed (Figure 1B). Until passage 2 or 3, the cocultured cells maintained their neuronal like morphology.

3.2. Proliferation analysis using MTT assay

Figure 2 shows the results of MTT assay for cell proliferation. The growth rate was determined on day 3,7 and 14 after seeding cultures. The higher proliferation rate was estimated in co-culturing conditions, whereas the hippocampal cells and BMSCs exhibited low proliferation in comparison to co-cultured cells. The cultures on day 14 showed a statistically significant difference between BMSCs and cocultured cells (***$p < 0.001$), and between cocultured and hippocampal cells (***$p < 0.001$).

3.3. Evaluation of neural potential of cultured cells

To examine the neurogenic potential of BMSCs, cocultured cells and hippocampal cells, the cells were immunostained for neuronal markers. The immunocytochemical staining revealed that the co-cultured cells were significantly immunoreactive for neural progenitor marker nestin and immature neuronal cell markers tubulin in comparison to only hippocampal cells and BMSCs respectively (Figure 3, Figure 4) However, the levels of tubulin was not significant in co-cultured cells in comparison to nestin. The expression of both markers in cocultured condition demonstrates that majority of the cells were differentiated into neuronal like cells and committed for neuronal lineage. The BMSCs shown negligible increase in expression of tubulin but slight expression for nestin (NPCS) was found validating neural stem cells existence in BMSCs cultures. This immunophenotypic and morphological modification in cocultured cells has maintained up to three passages in normal conditioned medium without any treatment of neurobasal media, EGF or neural-medium with N2/B27.

3.4. Expression of neuronal transcription factors in BMSCs, hippocampal and cocultured cells

After co-culturing of hippocampal cells with BMSCs up to passage 2, the expression of neuronal differentiation markers such as NeuroD1 and NGN-2 was evaluated by immunocytochemistry. The marked elevation of NeuroD1 and NGN-2 (Figure 5, Figure 6) was visible in hippocampal cells and cocultured cells. However, when we compared both the groups, the cocultured cells showed high intensity of NeuroD1 and NGN-2 thus indicating the greater affinity of cocultured cells towards neuronal differentiation. The data was also evaluated by comparison with expression of both markers in BMSCs (Figures 5 and 6). The expression of NeuroD1 and NGN-2 was hardly determined in BMSCs as compared to co-culture and hippocampal cells. The above observations suggest that immunophenotypic change is due to transformation of the cells towards neural lineage from mesenchymal and this might be achieved with support of hippocampal cells coculturing with BMSCs. Our findings also suggest that differentiation of BMSCs into neurons from co-cultures was regulated under control of neuronal transcription factors NeuroD1 and NGN-2 (see Figure 7).

4. Discussion

In the present study, the BMSCs were targeted for their differential capability into neural progenitor cells or neurons by treatment with different chemical compounds like β-mercaptoethanol or retinoic acid [33]. In addition, the approach of coculturing neurons with BMSCs has been formerly used to generate more neuronal like cells in comparison to chemical induction [33]. However, the generation of these lineages or differentiation of BMSCs into neurons are regulated under transcriptional regulation program or due to the activation of differentiating factors is still unknown. We have selected the important differentiation and transcription factors involved in neuronal induction settings i.e. NeuroD1 and NGN-2, and proposed to evaluate their role in coculturing of BMSCs with neuronal cells. In present study, we cocultured the hippocampal cells with BMSCs through direct cell-to-cell contact and observed rapid neuronal like morphology from cocultured cells as formerly reported [33]. In the present study, we have observed highest growth rate in cocultured cells thus indicating upregulation of transcription program or cell-to-cell support. The morphological evaluation shows healthy neuronal like cells from cocultured cells and it was comparable to the morphology of the hippocampal cells. In addition, the cocultured cells also maintained their neuronal like morphology and synaptic connections between cells with the passage of coculturing time. This sustained neuronal morphology might be due to true differentiation of BMSCs with the help of hippocampal cells as previous reports suggests that the cocultured of neuronal progenitor cells (NPCs) and BMSCs stimulates BMSCs to differentiate into neural stem cells and neurons [34]. In other protocols neuronal like morphology returns toward original cell morphology as chemical treatment discontinued [35]. After proliferation and morphological evaluation, we next examined the expression of

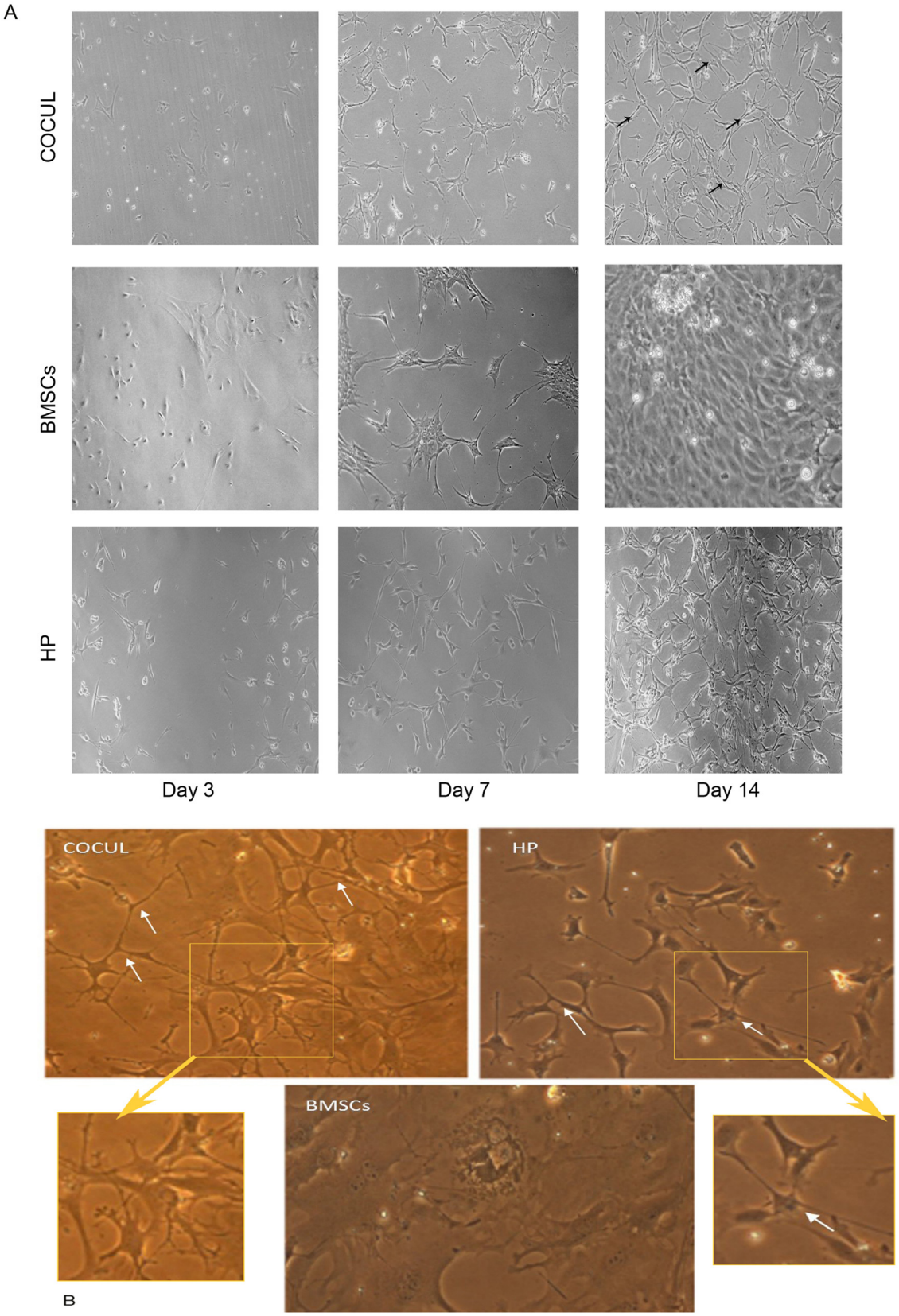

Figure 1. Morphological evaluation of cultured cells. (A)Representative photographs showing the morphological variations among BMSCs, hippocampal and co-cultured cells at different time periods. The arrow heads showed distinct synaptic connections and neuronal differentiation can be analysed in co-cultured and hippocampal groups at day 7 and 14. (B) Magnified images (20X) at Day 14 express prominent neuronal type morphologies with connections between cell processes. Arrow heads and enlarge section of COCUL and HP cells represents retracted cell bodies with synaptic linkages.

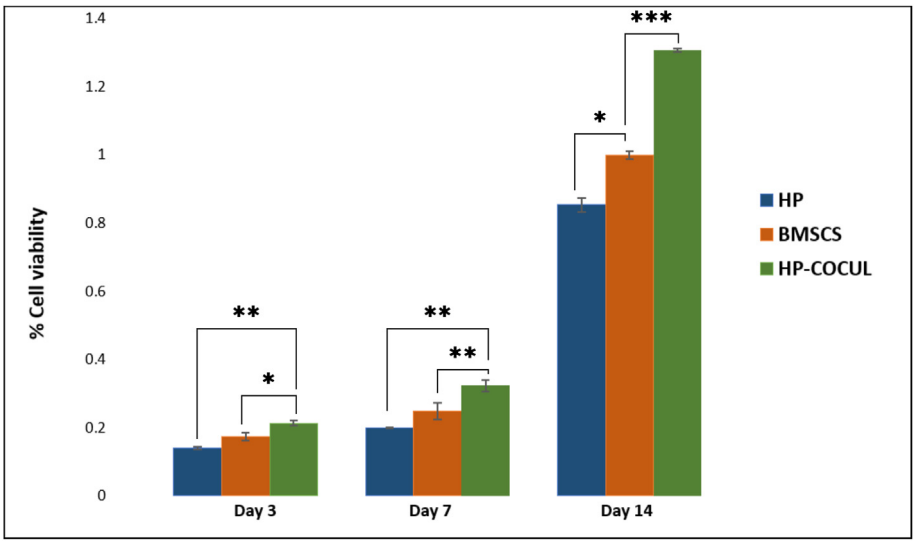

Figure 2. Growth proliferation of cells; The absorbance at histogram shows growth proliferation of cells following day 3,7 and 14. Significant difference between co cultured cells and BMSCs and between hippocampal and co-cultured cells was observed at day 3,7 & 14. The significance values are as *P < 0.05, **P < 0.01, & ***P < 0.001. Each bar represents mean ± S.E.M of three independent experiments.

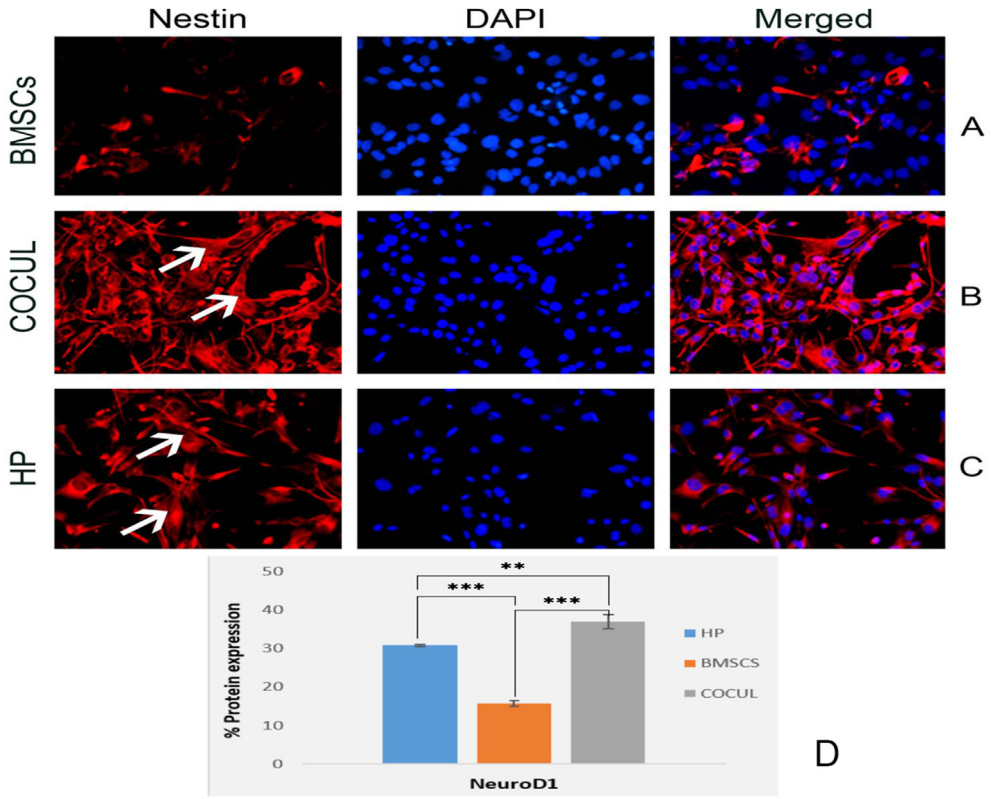

Figure 3. Immunocytochemistry of nestin in co-cultures, BMSCs and hippocampal cells. The arrows in images clearly show a marked increase in the immune fluorescence in co-culture group (panel B) as compared to the BMSCs (panel A), where small increase in expression of nestin also observed. Images were captured at 20 x magnification. Quantitative data shown as Panel D where Significant difference between co-cultured, BMSCs and hippocampal cells as determined by and *P < 0.05, **P < 0.01, ***P < 0.001.

neural progenitor markers nestin and tubulin for identification of immature neurons in all groups. The greater population of NPCs and immature neuronal cells in cocultured group demonstrated the differentiating potential of cocultured cells towards neural fate. These results are also in support of previous studies that reports the high expression of neuron specific enolase in the co-cultured cells, however, BMSCs does not distress the differentiation of NPCs [33, 34]. Many researchers have shown differentiation methods for BMSCs into neurons or neuronal like cells. Mostly it has been done by the use of retinoic acid, neural growth factors and chemical agents like BHA, β-mercaptoethanol (BME) and DMSO. Whether this neural differentiation was truly regulated under the neuronal induction or activation of neurogenic program in BMSCs has yet not studied. To recognize the relationship of BMSCs and NSCs during differentiation, we determined the role of two important neuronal transcriptional and differentiating regulators i.e. NeuroD1 and NGN-2. NeuroD1 strongly expressed in neuronal progenitor or neural stem cells going towards differentiation and promotes neurite formation. There are some evidences that NeuroD1 is involved in transcriptional regulation of bHLH protein, NGN. It has been reported that Wnt pathway augments NeuroD1 expression [36]. Our interesting results exhibit the significant

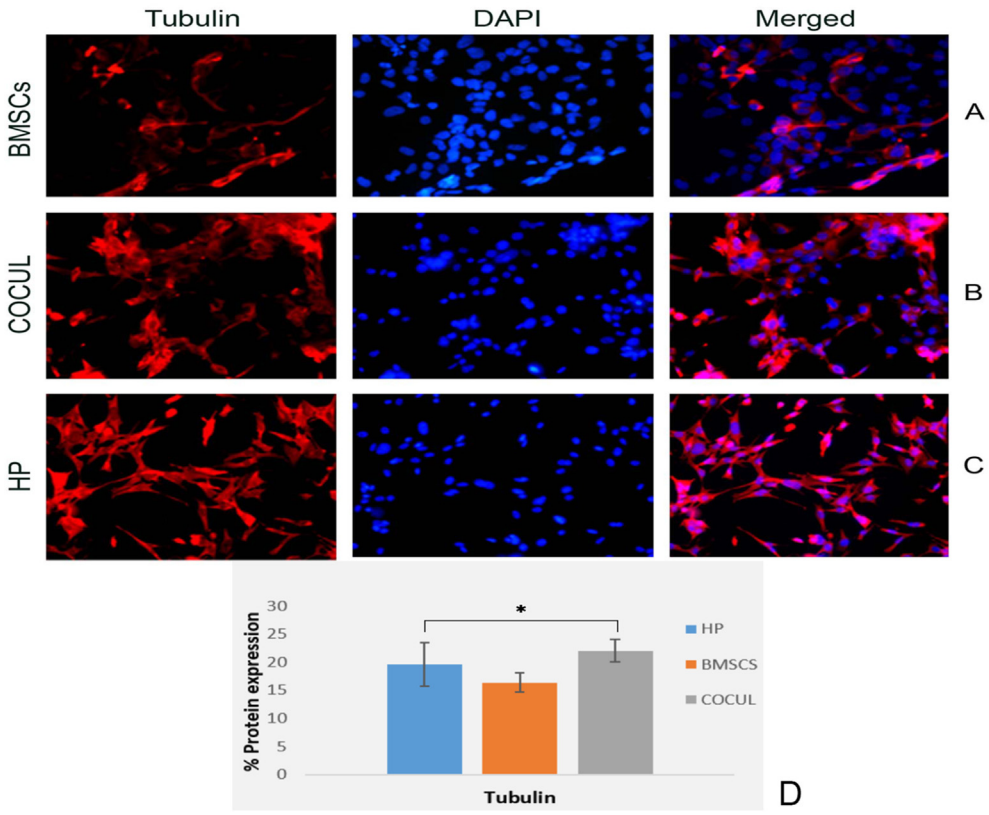

Figure 4. NPCs/BMSCS co-culture enhanced immunofluorescence of tubulin. Neuronal differentiation was demonstrated by increased level of tubulin expression in hippocampal cells (Panel A) and co-cultured cells (panel B). BMSCs did not express marked levels for tubulin. (D) Tubulin positive cells were counted in 5 blindly selected fields from from two wall chambers. Error bars reveal standard error of the mean of mean from three biological replicates Significant difference between co-cultured and hippocampal cells as determined by *P < 0.05.

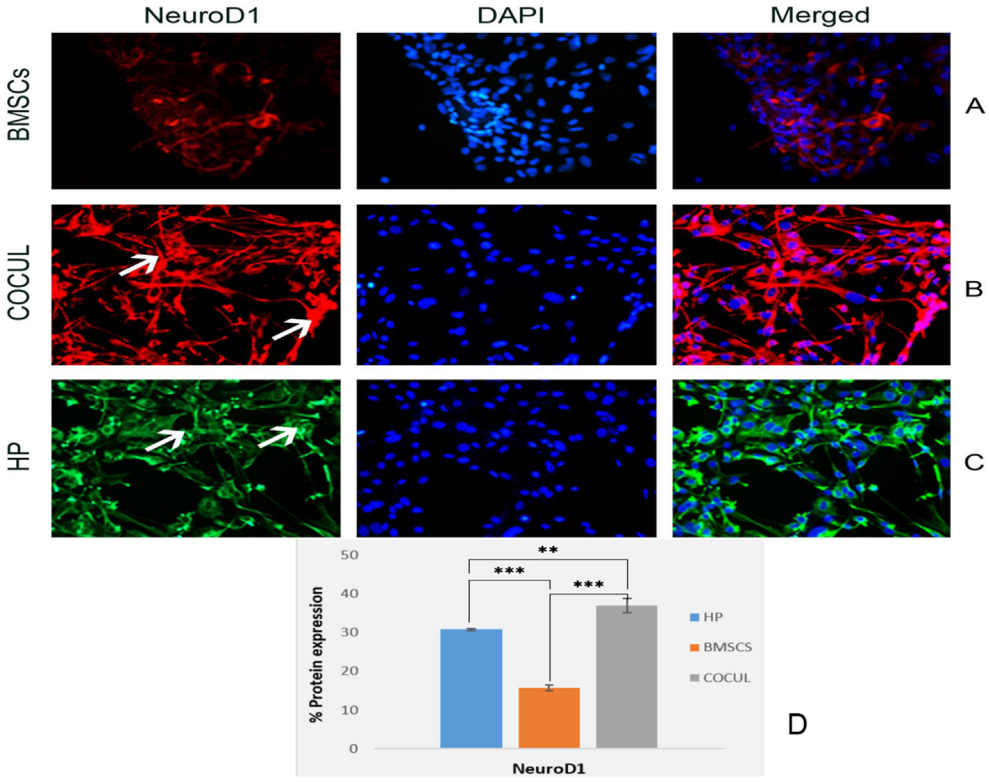

Figure 5. Neuro D1 is activated during co-culturing of BMSC with NPCs. Immunofluorescence images showing significantly upregulation of neurogenic differentiation factor 1 in co-cultured cells (panel B) comparable to panels A and C showing expressions in BMSCs and hippocampal cells respectively. Panel D show graphical illustration of % intensity (arbitrary unit) of NeuroD1 expression in COCUL, HP and BMSCs cells, where significant difference shown as **P < 0.01, ***P < 0.001.

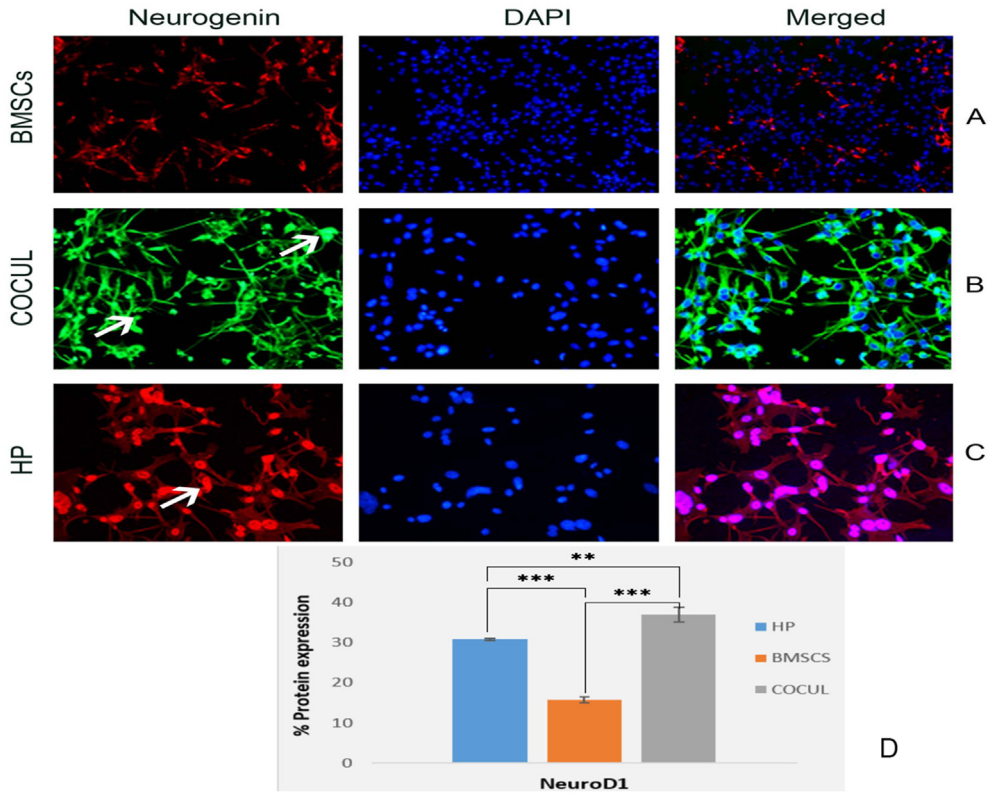

Figure 6. Co-cultures exhibit distinct immune expression of Neurogenin-2. Images in panel B and C showed significantly enhanced neurogenic differentiation is in co-cultured and hippocampal cells with elevated expressions of NGN-2 in comparison to BMSCs (panel A). Histograms (Panel D) represent elevated expression in coculture in comparison to BMSCS express as***P $<$ 0.001, and comparable expression between BMSCS and HP shown as**P $<$ 0.01.

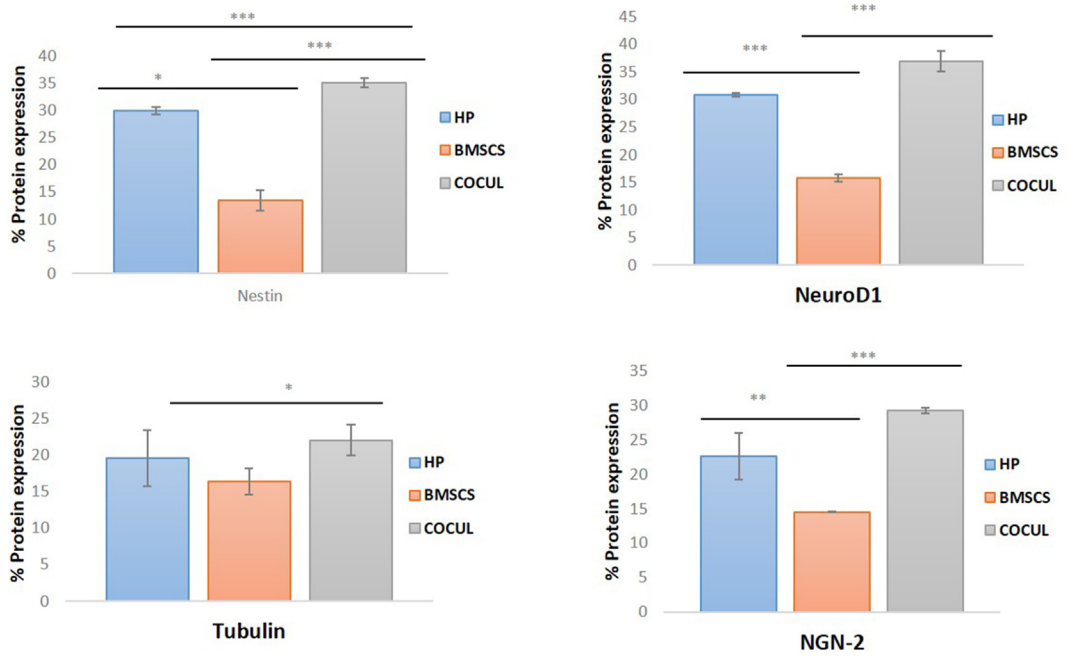

Figure 7. Levels of neurogenic differentiation markers (NeuroD1& Neurogenin-2), neural progenitor marker (nestin) and immature neuronal (tubulin) expressions in BMSCs, co-cultured and hippocampal cells. Error bars reveal standard error of the mean of 5 fields selected blindly from two-well chamber slides in three biological replicates and then % intensity (arbitrary unit) was calculated. Significant difference between co-cultured BMSCs and hippocampal cells as determined by and *P $<$ 0.05, **P $<$0.01, ***P $<$0.001 Between all groups, except tubulin all proteins showed significant upregulation in their expressions, although the marked elevation was determined in co-cultured cells comparative to BMSCs and hippocampal cells.

higher levels of NeuroD1 and NGN-2 in cocultured cells in comparison to only hippocampal cells. Additionally, the significant difference was found in expression of both transcription markers as compared to control BMSCs. The expression of NeuroD1 and NGN-2 in BMSCs has not been investigated previously and our results shows that the minor expression of neurogenic differentiating factors in BMSCs which suggest the existence of NSCs committed for neuronal lineage among BMSCs population. Previous data from co-cultures has shown that BDNF/NGF potentiate differentiation of NSCs by activating the Wnt/β and catenin signaling pathway and [37] it is evident that Wnt pathway triggers NeuroD1 expression ensuing initiation of neurogenic program. Taken together both these information we can speculate that the upregulation of NeuroD1 and marked proliferation of cells in co-cultures might be regulated through activation of Wnt pathway which requires further investigation to discover the exact mechanism. However, our results demonstrate that the differentiated neuronal progenitors or immature neurons in co-cultures are controlled under transcriptional activation of neurogenic differentiation.

In summary, the co-culturing of BMSCs with neonatal hippocampal cells promotes differentiation of BMSCS into neurons without disrupting the NPCs differentiation through activation of neuronal transcription factors NeuroD1 and Neurogenin.

5. Conclusions

These finding suggests that NeuroD1 and NGN-2, regulates differentiation of cells in BMSCs and hippocampal co-cultures and it open new avenues to treat the neurodegenerative disorders by transplantation/replacement cell therapy.

Declarations

Author contribution statement

Saba Majeed: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Aisha Aziz: Performed the experiments.

Shabana Simjee: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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