

PKA-GSK3 β and β -Catenin Signaling Play a Critical Role in *Trans*-Resveratrol Mediated Neuronal Differentiation in Human Cord Blood Stem Cells

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Abstract The role of resveratrol (RV), a natural polyphenol, is well documented, although its role on neurogenesis is still controversial and poorly understood. Therefore, to decipher the cellular insights of RV on neurogenesis, we investigated the potential effects of the compound on the survival, proliferation, and neuronal differentiation of human cord blood-derived mesenchymal stem cells (hCBMSCs). For neuronal differentiation, purified and characterized hCBMSCs were exposed to biological safe doses of RV (10 μ M) alone and in combination with nerve growth factor (NGF-50 ng). The cells exposed only to NGF (50 ng/mL) served as positive control for neuronal differentiation. The genes showing significant involvement in the process of neuronal differentiation were further funneled down at transcriptional and translational level. It was observed that RV promotes PKA-mediated neuronal differentiation in hCBMSCs by inducing canonical pathway. The studies with pharmacological inhibitors also confirmed that PKA significantly induces β -catenin expression via GSK3 β induction and stimulates CREB phosphorylation and pERK1/2 induction. Besides that, the studies also revealed that RV additionally possesses the binding sites for molecules other than PKA and GSK3 β , with which it interacts. The present study therefore highlights the positive impact of RV over the survival, proliferation, and neuronal

differentiation in hCBMSCs via PKA-mediated induction of GSK3 β , β catenin, CREB, and ERK1/2.

Keywords Resveratrol · Neuronal differentiation · Human umbilical cord blood stem cells · PKA GSK3 β / β -catenin signaling

Introduction

Neurogenesis is a natural process, comprising cellular proliferation, differentiation, and migration and is responsible for brain regeneration. However, several factors such as age, hormonal imbalance, chemical exposures, and injury are responsible for interrupting the natural process of neurogenesis and bring about severe neurodegeneration [1]. Oxidative stress is known to play a crucial role in neurodegeneration and is closely associated with neurodegenerative disorders [2]. Therefore, naturally occurring antioxidants are a popular choice for potent neuroprotectants. Supplementation of diet with antioxidants assures minimum side effects and increased health benefits unlike the synthetic drug consumption and medication. Resveratrol (RV) is one such natural stilbene polyphenolic compound found in grapes and red wine and is largely known for its antioxidant and anti-inflammatory properties that target the vascular and the central nervous system. RV has been widely reported to activate sirtuin 1 (SIRT1) and mimics the effects of calorie restriction, thereby playing a pivotal role in ameliorating the effects of neurodegeneration and improving the life span of several species [3]. The polyphenol compounds can easily cross the blood-brain barrier, and their beneficial roles have been thoroughly investigated in Alzheimer's disease and Parkinson's disease. RV is also known to delay and halt the progression of Huntington disease as well as brain ischemia [4]. It reportedly provides protection against hypoxic

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and toxic insults in ex vivo endothelial and primary neuronal cultures as well [5]. However, the role and underlying mechanisms of RV in the human umbilical cord blood-derived stem cell neuronal differentiation still need further investigation. Therefore, in the current study, we have tried to investigate RV-induced cascade mechanism involved in the neuronal lineages of human umbilical cord blood derived mesenchymal stem cells (MSCs). It has already been reported that one of the best-characterized cellular models used for studying neuronal differentiation pathways is the PC12 cell line, derived from rat pheochromocytoma cells [6].

Exposing PC12 cell line to nerve growth factor (NGF) leads to induction of neurite outgrowth and allows the differentiation of PC12 cells into sympathetic-like neurons [7]. These differentiated cells show typical characteristics of the peripheral nervous system such as expression of high molecular weight tau isoforms [8, 9]. The signaling pathway triggered by NGF in PC12 cells may activate Ras-dependent and independent cascades [10–12]. The Ras-dependent pathway includes sequential activation of Raf-1, MEK, and transient activation of ERKs [13]. Likewise, human mesenchymal stem cell derived from human umbilical cord blood also reportedly differentiates into neuronal cells in presence of NGF (50 ng/mL) [14]. Therefore, NGF is taken as positive control for the present study. As for the signaling molecules, it is reported that Wnt signaling is the leading pathway for neuronal induction in MSCs [15] and stem cells generally follow the Wnt signaling pathway for cell growth, development, and survival. In Wnt signaling, β -catenin plays a significant role in the induction of neuronal differentiation [16, 17]. β -catenin is regulated by upstream molecules like glycogen synthase kinase 3 beta (GSK3 β) [18]. GSK3 β , a proline-directed serine/threonine kinase, is a homodimer. GSK3 β phosphorylates β -catenin thus directing β -catenin towards ubiquitination and subsequently inhibiting neuronal differentiation [18, 19]. In our study, neuronal differentiation signals were initiated from protein kinase A (PKA), which is associated with neuronal induction in different cell lines [20–22]. cAMP-dependent protein kinase A (PKA) activation leads to growth of neuronal extensions and creates new synaptic connections [8]. However, its induction is dependent on cAMP, a secondary messenger which modulates the function of PKA, a regulator of neuronal differentiation. PKA is a heterotetramer containing two regulatory and two catalytic subunits, which get activated only when four domains of cAMP interact with binding sites of regulatory sub-unit resulting in detachment of catalytic subunits. PKA further regulates the downstream molecules for progressive neurogenesis. It has been hypothesized that MSCs differentiate into neuronal subtypes via protein kinase A (PKA) and Ca⁺²-mediated pathways [23]. In the previous studies, role of PKA in regulation of neurogenesis was directly associated with cAMP elementary binding protein (CREB) phosphorylation or activation [24]. However, the molecules

connecting the gap between PKA and CREB have not been discussed broadly in the previous reports. Therefore, the connecting key players of these molecules needed further investigation.

Through this study, we have tried to unravel the involvement of several important molecules participating in PKA-mediated neuronal induction by in vitro culture studies. We have, perhaps for the first time, reported the involvement of PKA and GSK3 beta in the suggested neuronal induction.

Materials and Methods

Reagents and Consumables

All the chemicals, reagents, and kits used in this study were purchased from Stem Cell Technologies and Sigma-Aldrich Corporation, unless otherwise stated. Recombinant human β -nerve growth factor (NGF) was purchased from PeproTech (Catalog No.450–01). Resveratrol (Catalog No.-R5010, sigma) was dissolved in culture grade dimethyl sulfoxide (DMSO) (Cat No. D2650, Sigma Aldrich) and stored at -20°C . Alpha minimal essential medium (α MEM) (Cat No. 12561056, Gibco), MSC qualified FBS (Cat No. 12662–029, Gibco), antibiotic solution (100 \times) (Cat No. 15240062, Gibco), GlutaMAXTM-I (100 \times) (Cat No.-35050-061, Gibco), sodium bicarbonate (7.5%) (Cat No. 25080-094, Gibco), fluorescent antibodies were purchased from Gibco (Invitrogen, Grand Island, NY, USA). Antibodies were purchased from Millipore (USA) and Chemicon International and D-PBS from Gibco (Cat No-14200-75, Gibco). Culture wares and plastic wares were procured from Nunc and Corning Inc. Autoclaved Milli-Q water was used in all the experiments.

Ethical Clearance for Collection and Transportation of Human Tissues

The entire study was carried out following the protocols and procedures approved by the Institutional Human Ethics Committees of CSIR-Indian Institute of Toxicology Research (CSIR-IITR) and CSM Medical University, Lucknow, India. The informed consent of parents was obtained before collecting blood from umbilical cord. Mothers enrolled in the study were of age ranging from 24.5 to 30 years for obtaining better quality and highly potent stem cells {Shen, 2011 #33}. They fulfilled the entire inclusion criteria and were free from malignancy or any other systemic disorder.

Processing of Human Umbilical Cord Blood

In total, 50 blood samples (40 mL/cord) were collected from the cord vein post-surgical delivery in a sterile container

having anticoagulant citrate dextrose (ACD) buffer and immediately used for further processing. A brief description of the protocol used to isolate the mononuclear cells from umbilical cord blood (UCB) is as under. Blood was diluted in the ratio of 1:1 with Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} , pH 7.5. Cells were isolated by density gradient centrifugation. The diluted blood samples were transferred to centrifuge tubes (50 mL) containing Ficoll-Paque solution and subjected to centrifugation at 2000 rpm for 30 min in order to isolate low-density mononuclear cells. Mononuclear cells were transferred to a new tube and washed twice with culture medium at 2000 rpm for 10 min, according to the protocol described by Erices et al. Cells were transferred in 25-cm² culture flask for further proliferation.

Proliferation of Mesenchymal Stem Cells

Expansion of MSCs was carried out following the method described [25] with some modifications pertaining to growth factors and cytokine concentrations. In brief, freshly isolated MSCs were cultured in plastic 25-cm² ultralow attachment culture flask (Nunc Incorporated, NY) at a density of 1×10^5 cells/mL in 5 mL of Alpha MEM supplemented with MSC qualified FBS (20%) and Glutamax (1%) (Invitrogen, Life technologies, USA), Penicillin 100 unit/mL, streptomycin 0.1 mg/mL, and sodium bicarbonate 2% (Sigma Aldrich). Cells were maintained as adherent culture in humidified chamber at 37 °C and 5% CO₂. After 24 h of culture, the growth medium was changed twice a week. The cells were monitored under inverted phase contrast microscope (Leica, Germany) every day and sub-cultured after reaching 85–90% confluence. The cells were treated with 0.25% Trypsin/EDTA and centrifuged at 800 rpm for 6 min. The pellet obtained was dissolved and re-seeded at a density of 1×10^7 nucleated cells/flask. The cultures were maintained as mentioned above. The cells were also characterized for specific surface marker via immunocytochemistry.

Characterization of MSCs by Flow Cytometer

Identification of isolated, cultured, and purified MSCs was carried out by fluorescence-activated cell sorting (FACS) analysis [25]. FACS was used for determination of purity of a population of MSCs by immunophenotyping of various CD markers present on the surface of stem cells. Freshly isolated as well as cultured MSCs at various passages were analyzed by two-color flow cytometry on a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Approximately, 1×10^5 cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-human CD90 and phycoerythrin (PE)-conjugated CD34 for 1 h at room temperature (RT) in dark. Post-staining, cells were washed thrice with $1 \times$ DPBS and finally re-suspended in 0.5 mL of $1 \times$ DPBS. At the

same experimental conditions, unstained cells, PE alone, and FITC alone stained cells were also run in parallel and served as control. The cells were gated on the cells with low FSC vs low SSC and population for CD90⁺ and CD34⁻ was selected. A total of at least 10,000 events for each sample were recorded at medium running mode. The actual percentage of pure population of CD90⁺ and CD34⁻ MSCs was analyzed with FACS Diva Software 6.0.

Identification of non-cytotoxic doses of resveratrol

Prior to starting the expression studies, non-cytotoxic doses of resveratrol (RV) were ascertained in MSCs using standard endpoints of cytotoxicity viz., MTT, NRU, LDH, Trypan Blue Dye Exclusion assays, and morphological alterations (preliminary study for dose selection). The entire studies in further experiments were carried out using selected non-cytotoxic dose of RV.

RV-Induced Neuronal Differentiation

MSCs were plated into 25-cm² flask at a density of 7×10^5 cells per flask and cultured for 24 h. Prior to exposure of cells to RV 10 μM, NGF 50 ng, RV + NGF (10 μM + 50 ng), they were incubated with low serum (2% FBS) medium for 24 h. Cells were exposed with RV, NGF, and RV + NGF, respectively for 4 days with a replacement of the exposure every alternate day.

Transcriptional Changes (Real-Time PCR) Transcriptional changes were studied in MSCs following exposure of RV both in the presence and absence of NGF. Briefly, total RNA was isolated from both experimental and unexposed control sets using Trizol (Invitrogen, USA). The purity and yield of RNA were assessed by Nanodrop ND-1000 Spectrophotometer V3.3 (Nanodrop Technologies Inc., Wilmington, DE, USA), and the quality was assessed by running RNA onto 2% denaturing agarose gel. Total RNA (1 μg) was reverse-transcribed into cDNA by Super Script III first strand cDNA synthesis Kit (Catalog No. 18080-051, Invitrogen Life Science, USA) and treated with RNase free DNase I to remove any potential DNA contamination. Quantitative real-time PCR (qRT-PCR) was performed by taqman dye (ABI, USA) using ABI PRISM 7900HT sequence detection system (Applied Biosystem, USA). Real-time reactions were performed in triplicate wells for each sample. The specificity for each primer was assessed by melting curve analysis and NITCs for respective primers viz. NF-M, βIII tubulin, synaptophysin. Actin-β was used as an internal control to normalize the data. Primer list is described in Table 1. The alterations in the expression of mRNA are expressed in relative quantification (RQ).

Table 1 The sequence of primers used in transcriptional studies

Name of gene	Forward	Reverse	Probe
NF-M	5'-CATAGTAAAGGAAGTCACCCAG-3'	5'-AATACAATGCTGGAGCCCC-3'	[6FAM]AACCCGTTCTGTTTGAAGCTGCC[TAM]
β III Tubulin	5'-TTTGGACATCTTTCAGGCC-3'	5'-TTTACACTCCTTCCGCAC-3'	[6FAM]CCTCCGTGTAGTGACCCCTTGGC[TAM]
PSD 95	5'-CCGCTACCAAGATGAAGACAC-3'	5'-TCCTCGTATTCATCTCCCC-3'	[6FAM]TCAACACAGATACCCCTAGAACGCCCA[TAM]
Nestin	5'-GGCACTTACCAGGACTTTG-3'	5'-GTTTTGGGTTGGTCACTGTC-3'	[6FAM]TCCCTACATTGGCTGTGAACCTTGGAC[TAM]
β -actin	5'-ACCTTCTACAATGAGCTGCG-3'	5'-CCTGGATAGCAACGTACATGG-3'	[6FAM]ATCTGGGTCACTTCTCGCGGTTG[TAM]
Synaptophysin	5'-CCTGTCTATTGCTCCATCCTG-3'	5'-GGCGTCTGGTTCACCTG-3'	[6FAM]AGCTGTCCACCCTCTTGAAAGGCTATG[TAM]

Translational Alteration (Western Blotting) MSCs were seeded in 75 cm² in normal serum medium for 24 h, then shifted to low serum (2% FBS) for 24 h, and finally exposed to RV in presence and absence of NGF for different time periods. On the completion of exposure, cells were washed with PBS, scraped in ice-cold PBS containing 1 mM sodium orthovanadate, 1% sodium fluoride, pelleted, and lysed using CellLytic Cell Lysis Reagent (Catalog no. C2978, Sigma, USA) in the presence of protease inhibitor cocktail (Catalog no. P8340- 5ML, Sigma-Aldrich Cooperation, USA), 1 mM sodium-orthovanadate, 1 mM DDT, and incubated for 30 min at 4 °C. The cell lysate was transferred to the micro-centrifuge tube and centrifuged (15,000g for 30 min) at 4 °C, and the cell lysate was carefully transferred to the micro centrifuge tube. The protein concentration was measured by the Bradford method (Thermo Scientific, USA) using bovine serum albumin as a standard. The protein obtained (40 μ g) was separated on 10% Tricine-SDS gel and transferred onto PVDF membrane (Millipore, USA) at 100 V for 2 h at 4 °C. The membranes were blocked at 4 °C in TBST blocking buffer (5% non-fat dried milk in TBS containing 0.1% Tween-20) for 2 h. Blots were incubated with the appropriate antibodies overnight at 4 °C. For neuronal markers, NF-M, β III tubulin, Nestin, and synaptophysin (each antibody is diluted in 1:1000 ratio) were used, and for study of signaling molecules, the antibody used was anti-phospho-TrkA (1:1000), anti-TrkA (1:1000), anti-phospho-ERK1/2 (1:1000), anti ERK1/2 (1:1000), anti-phospho-p38 (1:1000), anti-p38 (1:5000), anti-phospho-JNK (1:1000), anti-phospho-MEK1/2 (1:5000), anti-Rap1 (1:1000), anti-phospho-Raf1 (1:1000), anti-phospho-BRaf (1:1000), anti-phospho-PKA (1:1000), anti-phospho-GSK3 β (1:1000), anti- β catenin (1:1000), anti-phospho-CREB (1000) (Millipore, USA), anti-adenylate cyclase1 (1:500) (Abcam, UK), and anti- β -actin (1:5000) (Sigma, USA). After three washes with TBST, the membranes were re-incubated for 2 h at room temperature with secondary IR-labeled anti-primary immunoglobulin G (IgG) followed by image development using Licor Odyssey CLx and densitometry for protein-specific bands using Gel Documentation System (Alpha Innotech, USA) with the help of AlphaEase FC Stand Alone V. 4.0.0 software. β -Actin was used as an internal control to normalize the data. Exposure-induced alterations in protein expression were expressed in relative term fold change in expression by comparing the data with respective unexposed controls.

Following Western blot analysis, immunocytochemical studies were also conducted to further decipher the expression profile of the above-mentioned markers. After the completion of treatment duration, cells were fixed with 4% paraformaldehyde (PFA) (pH 7.4) for 30 min and then blocked for 1 h in a solution comprising bovine serum albumin (BSA) and Triton-X100 in 1 \times PBS. Following overnight incubation with primary antibodies, cells were washed three times with PBS to

remove the unbound antibodies. Then, fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse (1:500) and rhodamine-conjugated mouse anti-rabbit (1:500) antibodies were added to each well and kept on a rocker shaker in dark for 2 h at room temperature. Cells were then washed with PBS three times for 5 min each. Thereafter, the cells were visualized under an upright fluorescence microscope (Nikon Eclipse 80i equipped with Nikon DS-Ri112.7-megapixel camera, Japan) using specific filters for FITC and rhodamine. For each marker, 20 randomly selected microscopic fields were captured and analyzed for fluorescence intensity with the help of Leica Qwin 500 Image Analysis Software (Leica, Germany). The values were expressed in mean \pm SE of percent area for fluorescence intensity covered. The values of undifferentiated cells at day 0 were used as control to calculate the differentiation-induced alterations in the expression of each neuronal marker.

[Ca²⁺]_i Assay

The intracellular calcium ion levels have been demonstrated to play a key role in signal transduction and in various metabolic and physiological processes in the cells. Thus, even a small change in the level of intracellular calcium [Ca²⁺]_i may induce major alterations in the cellular activities. [Ca²⁺]_i was measured by fluorometric analysis using molecular probes fura-2 acetoxymethyl ester pentapotassium (fura-2-AM; Sigma-Aldrich, USA). MSCs were loaded with 3 μ M/L fura-2-AM in loading solution containing 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2 mM CaCl₂, 6 mM glucose, 25 mM Heps-NaOH (pH 7.4) buffer and incubated for 30 min at 37 °C in 5% CO₂ and 95% atmospheric air. Unbound fura-2-AM was removed by rinsing the cells twice with titration solution and saline A containing NaCl, 8.182 g/L; KCl, 0.372 g/L; NaHCO₃, 0.336 g/L; and glucose, 0.9 g/L. The cells (1 \times 10⁶ mL⁻¹) receiving RV (10 μ M) and/or NGF (50 ng/mL) or the unexposed control groups were represented in titration solution or saline A. Ca²⁺ fluorescence was measured by Synergy H1 hybrid plate reader (Biotek Inc., USA) at an emission wave length of 510 nm using a pair of excitation length at 355 and 380 nm, and (Fo) excitation was obtained at the ratio of that generated by 355/380. Fmax was obtained after cells were lysed with 0.1% Triton X-100. Fmin was obtained after the addition of EGTA (5 mM/L final concentration). Ca²⁺ fluorescence was examined simultaneously at time points 0, 20, and 40 min. The results shown are representative of three independent experiments carried out under identical experimental conditions. The concentration of intracellular calcium [Ca²⁺]_i was calculated using the following formula:

$$[Ca^{2+}] = Kd * \left\{ \frac{F_{\min 380}}{F_{\max 380}} \right\} * \left\{ \frac{R - R_{\min}}{R_{\max} - R} \right\}$$

where Kd is the dissociation constant of fura-2-AM calcium complex and its value is 224 nmol/L. The results are expressed in terms of nanomoles of calcium ion.

cAMP Assay

Cyclic AMP (cAMP), an intracellular second messenger, is involved in a marked number of cellular functions including neuronal differentiation in a gene transcription-dependent manner [26, 27]. For measurement of cAMP, firstly, MSCs were seeded in 6-well culture plates in normal medium for 24 h. The cells were then shifted to low serum (2% FBS) for 24 h and exposed to RV (10 μ M) in presence and absence of NGF (50 ng/mL) for different time periods. After removing the culture medium, the cells were incubated for 20 min in HCl (0.1 M) and scraped with a cell scraper. The cell lysates were centrifuged at high speed for 10 min, and the supernatant was used directly for the assessment of cAMP. The intracellular cAMP level was measured by the cAMP Direct Immunoassay Kit (Catalog No. ab65355, Abcam, UK) following the manufacturer's instructions.

Statistical Analysis Results were expressed as mean \pm standard error of mean (SEM) for the values obtained from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test using Graph Pad prism (Version 5.0) software.

Results

Isolation and Characterization of Stem Cells from Cord Blood

Human umbilical cord blood-derived adherent stem cells with a fibroblastoid appearance were grown on standard plastic surfaces. Surface cluster determinant (CD) marker expressions were identical to published reports on mesenchymal stem cells, i.e., CD34⁻ and CD90⁺. As a result, we refer to these cells as mesenchymal-like stem cells derived from human umbilical cord blood (hUCBMSC). To study expandability of cells, we established a growth profile of continuous passages up to 30 passages. In general, we observed a trend of decreasing passage density with increasing passage numbers (Fig. 1a). The trend of decreasing passage density indicates a general tendency towards slowing down of mitotic activity, possibly reflecting senescence and/or spontaneous differentiation. Immunocytochemical studies too (Fig. 1b) showed the presence of surface markers (CD90 and CD73). Fluorometric study revealed 80% population of pure MSCs (Fig. 1c).

Fig. 1 Isolation and characterization of HUCB-MSCs. **a** Phase contrast picture of cells grown in control medium (MEM- α 20% MSC qualified). **b** Marker expression for the MSCs for surface marker CD90⁺ marker (green) CD73⁺ (red), nuclei were counterstained with DAPI (blue). **c** FACS analysis showing the presence of 80.6% population of CD90⁺ surface marker in MSCs conjugated with FITC in comparison to unstained control

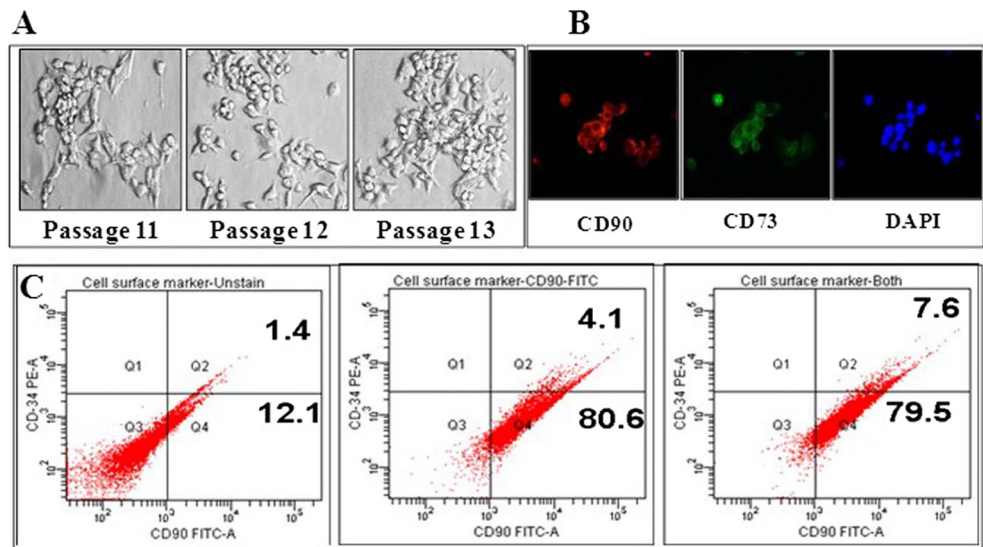
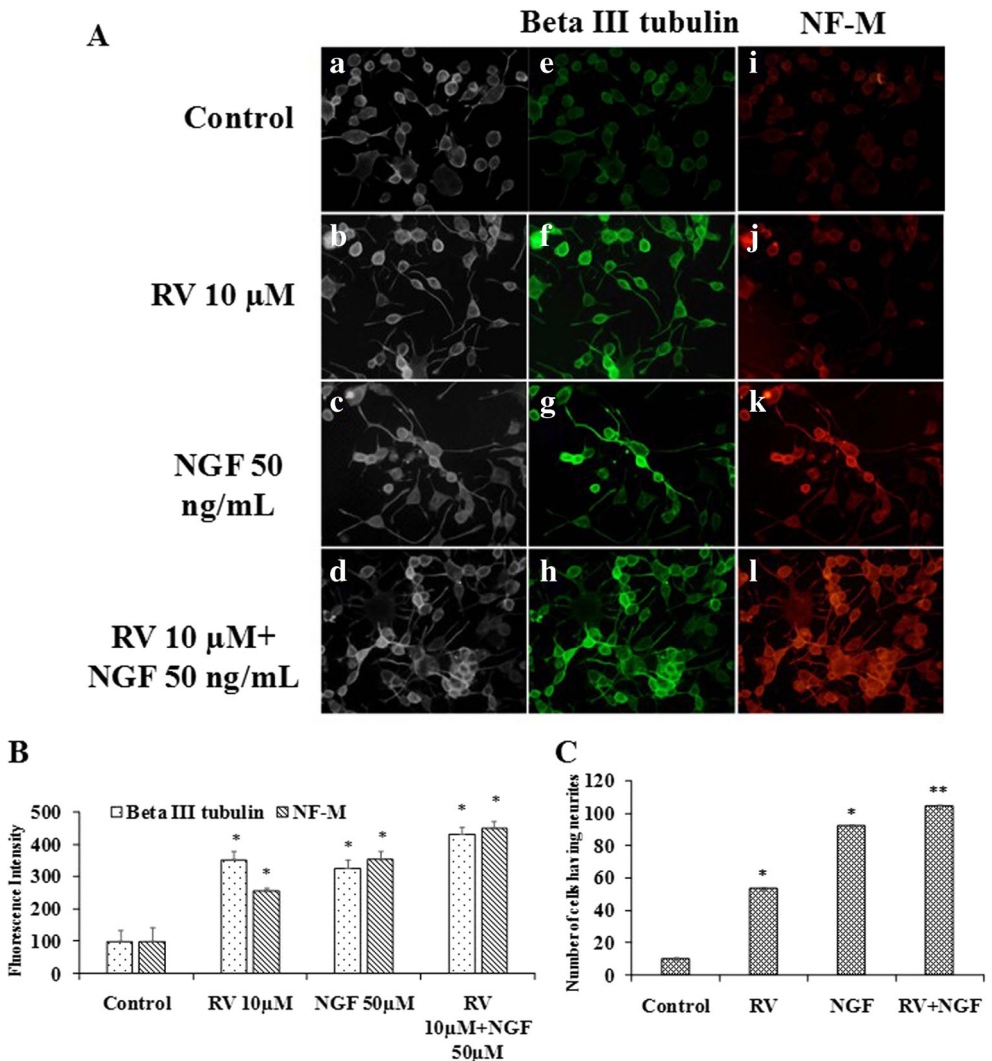


Fig. 2 Neuronal differentiation of MSCs: **a** morphological RV, NGF, RV + NGF dependent aspects of neuronal differentiation, **b, c, d** phase contrast views revealing the general morphology of cells under different treatment conditions. Cells were stained for beta III tubulin (**e, f, g, h**) and NF-M (**i, j, k, l**) early and late neuronal markers, respectively; **a, e, i** are control cells without treatment. Neither neurite like outgrowth nor upregulation of markers' gene expression is observed in control cells. Cells after 4 days of treatment with RV10 μ M induce neurite like outgrowth of cells as well as expression of neuronal markers, beta III tubulin (**f**) and NF-M (**j**). Cells treated with NGF 50 ng/ml neurite like outgrowth as well as marker gene expression are up-regulated. **g, k** Cells treated with (RV10 μ M + NGF50 ng/ml) show that highest number of neurites and neurite bearing cells were observed (**b**). The change in expression pattern is statistically significant as indicated by $P < 0.01$. **c** Percent intensities were calculated by ImageJ 1.48v, Nikon, NIS, USA



Neuronal Differentiation Induced by RV

We analyzed hUCB-MSCs for their neural differentiation potential by incubating cells in RV (10 μ M) and NGF (50 ng/mL) alone as well as in combination. On day 4, we observed morphological changes of cells as compared to the control (Fig. 2a) such as rounding-up of cells and development of neurite-like extensions in more than 50% of the population (Fig. 2b). Quantification of expression of various neural marker was achieved by immunocytochemistry, and the results demonstrated that NF-M expression in case of RV-exposed MSCs was as 256 ± 6.99 , in NGF 50 ng exposed cells was 355.40 ± 23.65 and in combination was 451.10 ± 21.55 at day 4. β III tubulin expression in case of RV-exposed MSCs was as 353 ± 25.10 , in NGF 50 ng/mL exposed cells was 326.01 ± 27.39 , and in combination was 432.10 ± 21.46 at day 4. Neuronally differentiated cells have more numbers of neurite bearing cells in RV-exposed cells (Fig. 2c).

Transcriptional study shows the involvement of early and late neuronal markers in the differentiation process. In RV-exposed cells, the expression of nestin, β III tubulin, NF-M,

and synaptophysin was 1.9-, 1.5-, 1.99-, and 2.0-fold, respectively. Co-exposed cells have shown additive effect in expression of neuronal markers. Expression of Nestin, β III tubulin, NF-M, and synaptophysin was found to be 3-, 3.2-, 4.3-, and 4.9-fold, respectively (Fig. 3a). Same increase in expression pattern of neuronal markers was obtained through Western blotting. β III tubulin, NF-M, Nestin, and synaptophysin had 1.29-, 1.13-, 1.63-, and 1.36-fold change value in RV-induced neuronal induction, respectively. RV shows an additive effect with NGF. β III tubulin, NF-M, Nestin, and synaptophysin underwent 1.93-, 2.37-, 5.17-, and 2.81-fold change, respectively (Fig. 3).

Biochemical Analysis of Key Molecules Involved in Neurogenesis

For confirming the molecules involved in neuronal differentiation induced by RV, we carried out translational studies (Fig. 4). The data shows cAMP and Ca^{++} -dependent activation of PKA. PKA is the molecule which plays a key role in RV-induced neuronal differentiation of hUCB-MSCs. It is demonstrated that RV

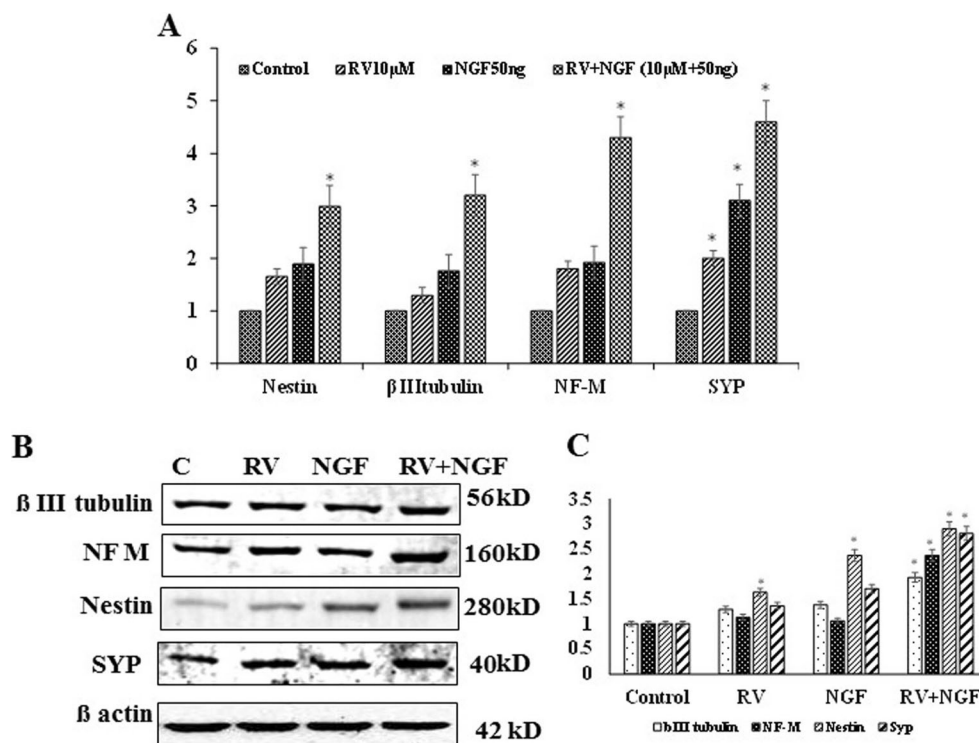


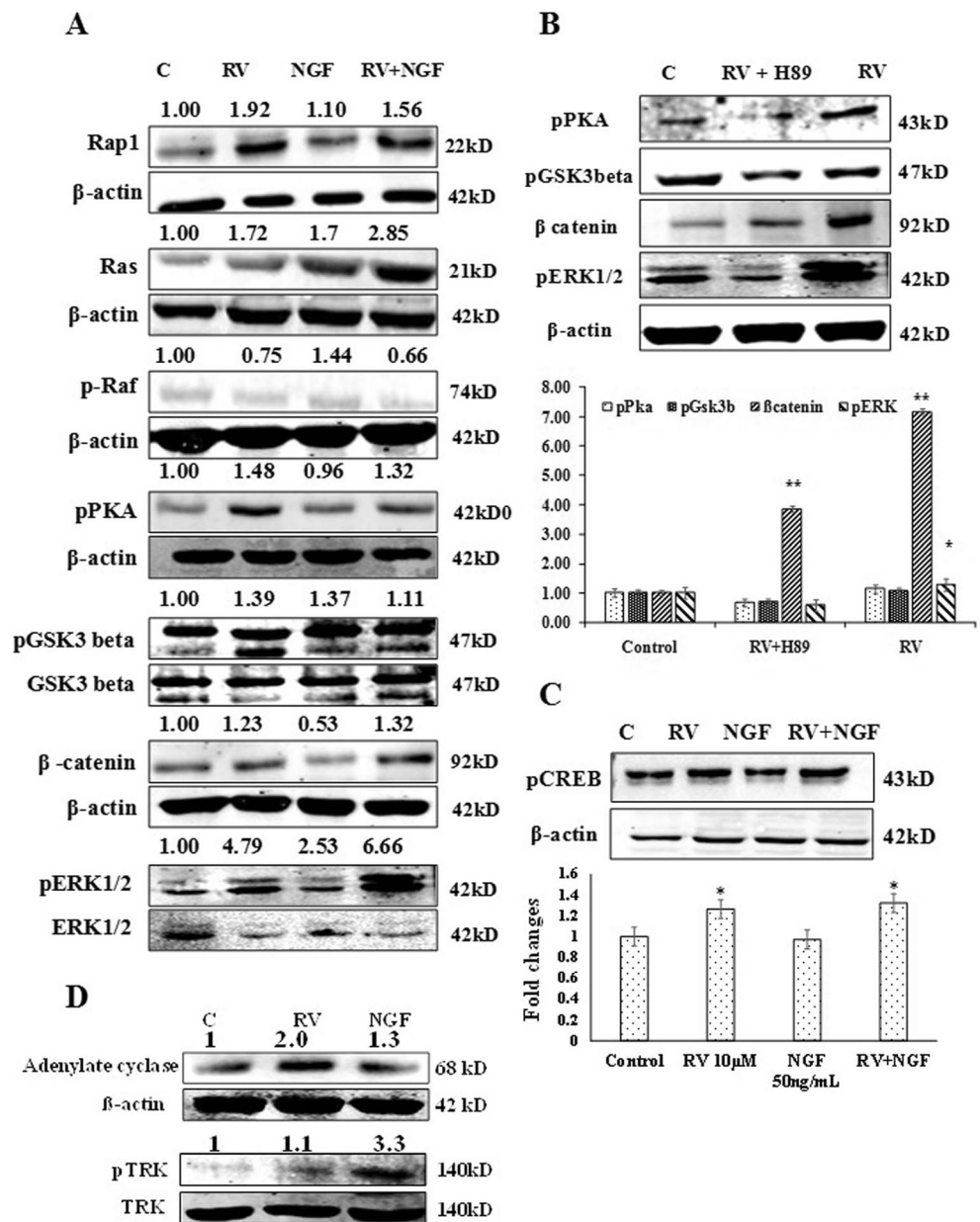
Fig. 3 Expression of neuronal markers in HUCB-MSCs. **a** Cells were differentiated as described in material and method section. RNA was isolated on day 4. The expression level of genes was determined by RT-qPCR. Data presented were normalized with respect to β actin. Significant differences are indicated by Dunnett test. **b, c** Biochemical analysis of neuronal markers induced by RV. Western blot analysis showing the expression of neuronal markers, presence of early as well as late neuronal markers indicates mature and complete neuronal

differentiation induced by RV, NGF, RV + NGF. Fold changes: the relative protein level was calculated according to the densitometry of each band, normalized according to the internal control. The control group was arbitrarily set at 1.0 for ease of comparison for normalization. Quantification was done in Gel Documentation System (Alpha Innotech, USA) with the help of AlphaEase FC StandAlone V.4.0 software

activates PKA by activating Rap1. Ras and Raf molecules were not found to be involved in PKA activation; however, these two molecules are otherwise important for signal transduction. PKA phosphorylates GSKIII β , beta catenin, and ERK1/2 simultaneously in Fig. 4a. The connectivity of above molecules was also confirmed by inhibitor studies. When PKA inhibitor: H89 (15 μ M) was introduced to these differentiating cells (Fig. 4b), the expression of GSK III β , beta-catenin, and ERK1/2 downregulated (Fig. 4c) thus depicting the involvement of CREB in RV-induced neuronal differentiation. The involvement of TRK with NGF is shown in Fig. 4d. TRK is found to be significantly overexpressed while in RV-exposed cells, the expression of TRK was observed but not significant. However, adenylate cyclase upregulation is effectively associated with RV exposure in Fig. 4d.

Role of Ca²⁺ and cAMP in Neuronal Differentiation
 Calcium signaling is involved in many different intracellular and extracellular processes ranging from synaptic activity to cell-cell communication and adhesion [28]. In the brain, calcium is essential for the control of synaptic activity and memory formation, a process that leads to the activation of specific calcium-dependent signal transduction pathways and implicates key protein effectors, such as CaMKs, adenylate cyclases, cyclic-AMP, MAPKs, and CREB [29, 30]. To determine the activation of intracellular calcium levels [Ca²⁺]_i, cells were treated with RV in the presence and absence of NGF. In RV exposure groups at t0 time point, the value was 87.25 \pm 9.53 which increased to 91.00 \pm 9.61 at t40 while in NGF-exposed group, it was 85.50 \pm 3.69 and 87.75 \pm 6.23 at t0 and t40, respectively, and in RV + NGF exposure group, these values were 85.00 \pm 21.76 and

Fig. 4 **a** Western blot analysis, Rap1 dependant activation of PKA which is associated with phosphorylation of adjacent downstream molecules GSK3 β , β -catenin, and ERK1/2. **b** Inhibitor studies also support the role PKA in phosphorylation of downstream molecules GSK3 β , β -catenin, and ERK1/2. **c** RV 10 μ M upregulate CREB phosphorylation. **d** Involvement of TRK and adenylate cyclase activity with NGF and RV, respectively, are described. pTRK is not activated by RV 10 μ M but with NGF 50 ng, while adenylate cyclase is activated by RV 10 μ M. Fold changes: the relative protein level was calculated according to the densitometry of each band, normalized according to the internal control. The control group was arbitrarily set at 1.0 for ease of comparison for normalization. Quantification was done in Gel Documentation System (Alpha Innotech, USA) with the help of AlphaEase FC StandAlone V.4.0 software



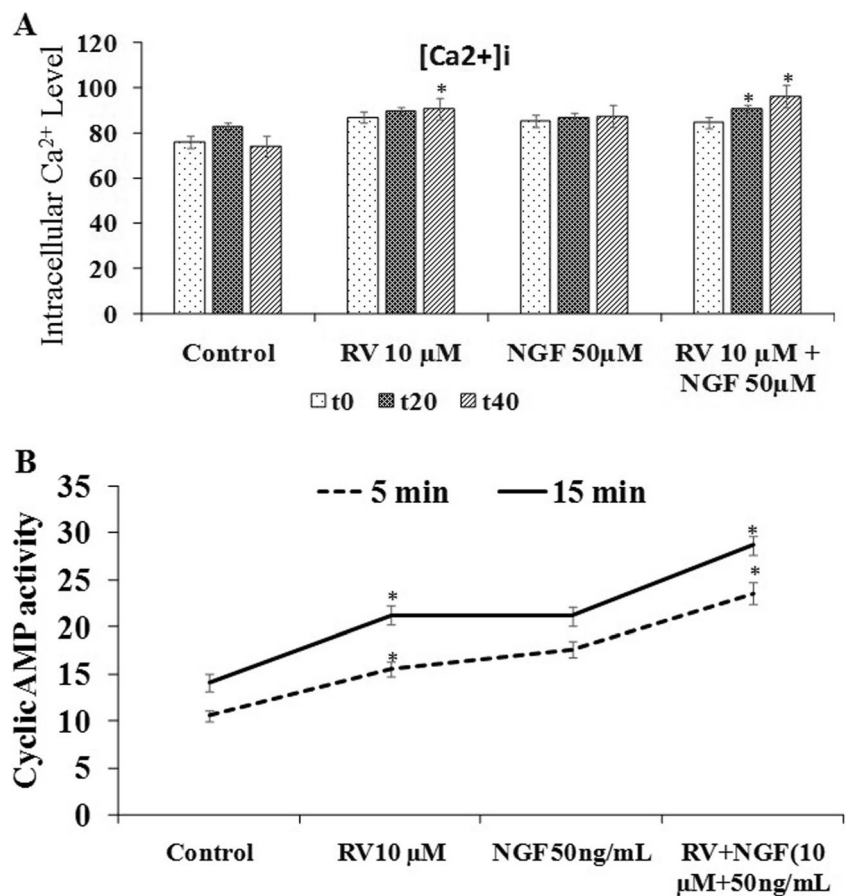
96.17.54, respectively. From the above data, it can be concluded that in case of NGF, the change in intracellular calcium ion is meager as compared to that of RV and RV + NGF groups depicting their role in calcium ion release in neuronal cells (Fig. 5a). Even though the results reported here are not significant, the possibility of involvement of Ca^{+2} modulating the activity of other second messengers for other cellular functions cannot be ruled out. Cyclic AMP, a secondary messenger, modulates cell growth and differentiation in organisms from bacteria to higher eukaryotes [31]. To determine the cyclic-AMP activity, MSCs were treated with RV or/and NGF. After 5 min, the value was recorded in control cells, i.e., 10.56 pmol/mg protein, and exposure of cells to RV, NGF, and combination of both significantly upregulates the levels of cAMP, i.e., 15.53, 17.30, and 23.60 pmol/mg protein, respectively. The maximum levels of cAMP were recorded at 15 min; the levels in control, RV, NGF, and RV + NGF were 14.30, 21.30, 21.60, and 28.70 pmol/mg protein, respectively, in Fig. 5b.

Discussion

The developing nervous system requires several growth factors and other stimuli which together regulate a variety of processes such as the proliferation of neurons, growth survival, and

synaptic connectivity of developing neurons [9, 32, 33]. Some stimuli involved in these processes are capable of transducing signals directly into the nucleus to influence the nuclear gene expression [34]. In vivo rodent animal models have been largely employed for the neurodevelopmental studies, but human-based studies are somewhat underrepresented. Ethical constraints and lack of samples restrict the progress of human-based research. However, in vitro studies employing cells and cell lines of human origin offer a good alternative in this regard. In our current study, we used human umbilical cord blood derived mesenchymal stem cells to investigate the differentiation and associated pathways involved in the process of neuronal induction. Mesenchymal stem cells are multipotent in nature and easily differentiate into the neuronal subtypes and thus serve as one of the best available in vitro models for neuronal/regenerative and developmental studies [35, 36]. The role of nerve growth factor in the neuronal differentiation of human umbilical cord blood-derived mesenchymal stem cells is well reported [14]. Polyphenolic compound RV at a concentration of 10 μM was found to be appropriate for neuronal induction in human umbilical cord blood-derived MSCs. Mechanism of signaling, required for neuronal induction for MSCs, is well reported in literature [37–39]. Our study showed that when cells were treated with RV containing medium, a set of molecular events for neuronal differentiation got triggered.

Fig. 5 **a** Fluorometric analysis of intracellular calcium levels in HUCB-MSCs induced by RV in presence and absence of NGF. Intracellular calcium was measured in MSCs using Fura-2 AM dye. Values are mean \pm SEM of three experiments each carried out in triplicate. **b** RV promotes the cyclic-AMP activity in MSCs. The cells were seeded in 6-well plates in normal medium for 24 h. The cells were then shifted to low serum (2% FBS) for 24 h and exposure to RV (10 μM) in presence and absence of NGF (50 ng/mL) for different time periods (5 and 15 min). Values are mean \pm SEM of three experiments each carried out in triplicate



Experiments were carried out up till 16 days (data not shown), but accurate differentiation was observed at day 4. Therefore, 4 days is taken for experimental analysis. Neurite length study was conducted for identification of neuronal induction. It was found that RV was able to induce MSCs into neurons. Marker analysis showed that as differentiation proceeded, there was a gradual decrease in the markers of stemness and an increase in the expression of neuronal markers. Differentiated MSCs expressed beta III tubulin, NF-M, Nestin, and synaptophysin. The presence of these early and late neuronal markers clearly supports that successful neuronal differentiation was achieved. During the study, NGF at a concentration of 50 ng/mL was taken as a positive control for neuronal induction {Jahan, 2017 #32}. Neurotrophic signaling pathways regulate a wide range of nerve cell functions including neural differentiation, axonal and dendritic growth, and survival along with various aspects of learning and memory [40, 41]. NGF induces neuronal differentiation via TRK receptor following the Raf/Ras pathway. Although signaling through the TRK-receptor-tyrosine-kinase-family is normally associated with biological processes like cell survival, growth, and development [42, 43], its involvement in neuronal differentiation is also reported. In PC12 cells, RV exposure has been shown to stimulate differentiation which results in a sympathetic response like neuronal cell phenotype characterized by neurite elongation

[44]. In our study, we have investigated the role of some of the key signaling molecules associated with the mechanism of neuronal induction. These molecules have been found to be either directly involved or indirectly involved.

It was clearly observed that PKA is responsible for GSK3 beta phosphorylation and thus inhibits the β -catenin degradation and is thus responsible for the activation of CREB. Phosphorylation of GSK3 beta at its serine residues results in inactivation of the enzyme but not in signaling activation [45]. The inactivation of GSK3 beta results in the stabilization of its downstream target beta catenin which is otherwise ubiquitinated and degraded in the proteasome [46]. GSK3 beta plays an important role in cellular differentiation in *Xenopus laevis*, *C. elegans*, and *Drosophila melanogaster* by regulating body axes or body segmentation [47]. It is reported that GSK3 beta has a role in neuronal proliferation and differentiation regulating the neuronal survival and activation of the transcripts of a set of neurogenesis related target genes/proteins [48]. It has been reported in a previous study that GSK3 beta inhibited by SB216763 decreases viable cell numbers. Reduced number of viable cells within the inhibited group could have two explanations, first, the cells stop proliferating and begin to differentiate or second, the cells may die either by apoptosis or necrosis [48]. The involvement of GSK3 beta, β -catenin, and ERK1/2 was further confirmed with the inhibitor study. When PKA was inhibited with 15 μ M (H89), all the

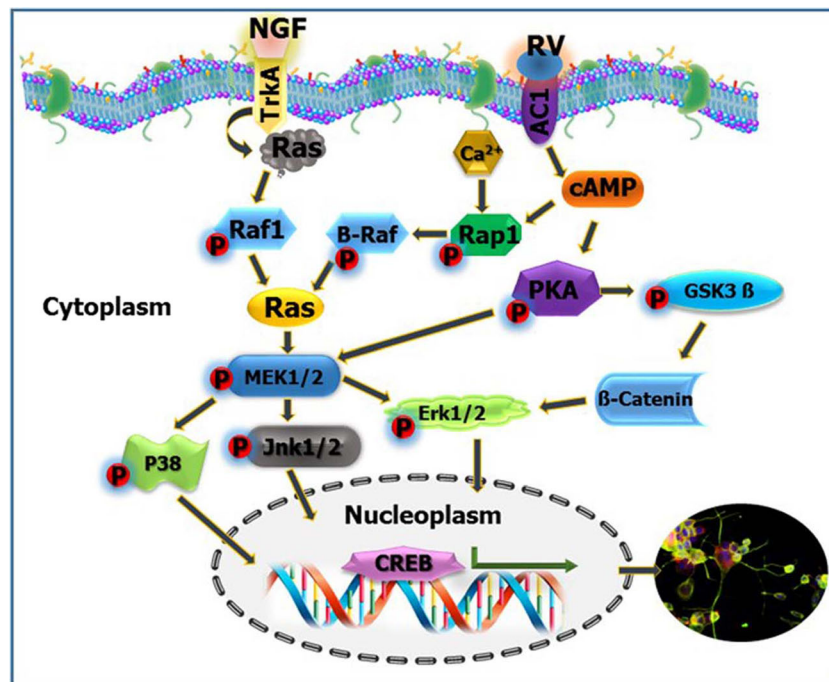


Fig. 6 Proposed schematic representation depicting the signaling mechanism by which RV posed the additive response to the activity of NGF to induce neuronal differentiation in MSCs. The RAS/RAF/MEK/ERK pathway(s) are activated by nerve growth factor (NGF), which binds to the receptor tyrosine kinase (RTK) and leads to the activation of the small G-protein RAS. Subsequently, RAF, MEK, and ERK are activated in a cascade of phosphorylation events. Through the phosphorylation of many targets, MAPKs regulate cell fate. RV leads to the

activation of adenylyl cyclase and increases the levels of intracellular calcium, activated adenylyl cyclase, which are involved in the conversion of ATP into cAMP. The second messenger cAMP acts via various effectors and poses number of cellular effects. The intracellular calcium plays a critical role to increase the levels of the small G-protein, Rap1 via Rap1/B-Raf signaling complex. These two pathways interconnect or crosstalk at MEK1/2. This allows cells to regulate the distribution, duration, intensity, and specificity of the response

down-stream molecules got inactivated in Fig. 4b. In neurons, an increase in the intracellular Ca^{+2} is largely brought about by voltage or ligand-gated cation channels [49]. Upon membrane depolarization, Ca^{+2} influx occurs and interacts with a large number of intracellular molecules [50]. One of the most well-characterized molecules, i.e., Ca^{+2} binding protein calmodulin (CaM), is known to be responsible for the cellular activities [51]. In this study, RV (10 μM) was able to release the Ca^{+2} in an appropriate quantity to induce cellular activity at 40 min (Fig. 5a). RV-induced Ca^{+2} release increased in a time-dependent manner, while NGF (50 ng/mL) induced a steady release of Ca^{+2} . It is reported that activation of cAMP/PKA, Rap1, and release of intracellular calcium ions are required for the NGF-induced neurite outgrowth in PC12 cells [26, 52]. Intracellular cAMP is enzymatically produced from ATP by adenylyl cyclases, either by transmembrane adenylyl cyclase (tmAC) or by soluble adenylyl cyclase (sAC) [53]. NGF after binding to TrkA activates sAC to produce cAMP [54]. Subsequently, activated PKA and Epac converge to activate Rap1 [55], the mentioned mediator of sustained MAPK activity [10]. In contrast, Forskolin (FK) binds tmAC to produce cAMP and activates PKA and Braf/Rap1 in PC12 cells. While FK stimulation depends on the activation of the MEK/ERK signaling but are PKA independent in the PC12 cells, regulation of cAMP and the activity triggered by RV in hUCBMSCs appeared to be quite important for the different general neuronal process such as axonal guidance, neuronal transmission, and neuronal synaptic plasticity.

The kinase responsible for phosphorylation event was identified as the cAMP-dependent protein kinase (PKA) [56]. PKA activity is regulated by molecules that alter cAMP level and the molecule upstream of cAMP is adenylyl cyclase [57]. In the nervous system, G protein coupled receptor neurotransmitters and neuro peptides can couple and interact with cAMP and PKA, ultimately activating CREB [58]. CREB activation via PKA can also occur in response to the many other stimuli capable of increasing intra-cellular Ca^{+2} levels, and increased Ca^{+2} also plays a key role in CREB activation [59, 60]. Neuronal growth factors appear to have the capacity to trigger parallel pathways leading to CREB phosphorylation through the activation of a single receptor. Many such growth factors including neurotrophins signal via receptor tyrosine kinase which upon ligand binding and dimerization activate several known signaling cascades [61]. One of the best characterized is the Ras/ERK pathway, a cascade of kinases which once triggered ultimately results in the phosphorylation of CREB and phosphorylation of CREB results in neuronal differentiation [62]. The Ras/ERK dependent phosphorylation of CREB can be carried out by different kinases. However, in MAPK pathways, receptor tyrosine kinases activate a second major signaling pathway, the PI3K kinase/AKT pathway for which routes to CREB, are well documented [63]. There is now evidence to indicate that in some specific situation, PI3K pathway is important for CREB activation (Fig. 6). We have perhaps, for the first time, defined the relation of GSK3 beta and PKA in neuronal induction via RV.

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Compliance with Ethical Standards

Conflict of Interest Authors of this manuscript have no conflict of interest among them or anybody else regarding the scientific contents, financial matters, and otherwise.

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