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Simulated Microgravity Promoted Differentiation of Bipotential Murine Oval Liver Stem Cells by Modulating BMP4/Notch1 Signaling

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ABSTRACT

Faster growth and differentiation of liver stem cells to hepatocyte is one of the key factors during liver regeneration. In recent years, simulated microgravity, a physical force has shown to differentially regulate the differentiation and proliferation of stem cells. In the present work, we studied the effect of simulated microgravity on differentiation and proliferation of liver stem cells. The cells were subjected to microgravity, which was simulated using indigenously fabricated 3D clinostat. Proliferation, apoptosis, immunofluorescence assays and Western blot analysis were carried out to study the effects of simulated microgravity on liver stem cells. Microgravity treatment for 2 h enhanced proliferation of stem cells by twofold without inducing apoptosis and compromising cell viability. Analysis of hepatocyte nuclear factor 4-α (HNF4-α) expression after 2 h of microgravity treatment revealed that microgravity alone can induce the differentiation of stem cells within 2–3 days. Probing bone morphogenic protein 4 (BMP4) and Notch1 in microgravity treated stem cells elaborated downregulation of Notch1 and upregulation of BMP4 after 2 days of incubation. Further, blocking BMP4 using dorsomorphin and chordin conditioned media from chordin plasmid transfected cells attenuated microgravity mediated differentiation of liver stem cells. In conclusion, microgravity interplays with BMP4/Notch1 signaling in stem cells thus inducing differentiation of stem cells to hepatocytes. Present findings can be implicated in clinical studies where microgravity activated stem cells can regenerate the liver efficiently after liver injury. J. Cell. Biochem. 112: 1898–1908, 2011.

KEY WORDS: BIPOTENTIAL MURINE OVAL LIVER STEM CELLS; BMP4; DIFFERENTIATION; MICROGRAVITY; NOTCH1

Liver being an important organ of the body has regenerative capacity. However, chronic liver diseases marginalize the regenerative capacity of liver that leads to fibrosis, and eventual development of cirrhosis. Currently the only curative treatment for advanced liver cirrhosis is liver transplant. Although liver transplant has become a procedure with a relatively good 5-year survival, organ donation has not kept up with demand. This has resulted in an increasing number of patients on the liver transplant waiting longer for a donor organ, which leads to increased morbidity and mortality. Beyond any doubt, advanced liver disease demands alternative treatment strategies and cirrhosis stands first in the line. The present study aims to develop potential strategies in using stem cell therapies for liver regeneration [Forbes, 2008].

The oval cell is a blast-like, liver-specific stem cell, capable of self-renewal and multipotent differentiation. Bipotential murine oval liver (BMOL) stem cells are 10 μm sized hepatic oval cells having the potency to differentiate to either hepatocytes or cholangiocytes depending on inductive signal. However, the ability of these cells to become functional hepatocytes remains unknown. These cells do not originate in bone marrow but in the liver itself and they have valuable properties for therapeutic liver repopulation [Wang et al., 2003]. Oval cells have been identified in the regenerative areas in patients with chronic liver injury or sub-massive hepatic necrosis [Herrera et al., 2006]. Oval cells being hepatocyte progenitors have also been of interest in the field of liver cell transplantation and therapeutic liver repopulation [Grompe et al., 1999; Shafritz et al., 2001; Lowes et al., 2003; Fausto, 2004].

Microgravity is a condition in which effect of acceleration due to gravity is not experienced, and hence the subjects will be in a state of float, creating an environment called weightlessness or zero gravity

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environment. It has been reported by various groups that microgravity promotes proliferation and differentiation of stem cells [Lelkes et al., 1998; Herrera et al., 2006; Ko et al., 2007], but its inductive implications in BMOL has not been studied yet.

Notch signaling is an evolutionarily conserved system for cell–cell communication. The Notch receptor is a single transmembrane spanning protein that receives signals from cell-bound ligands of the Delta or Serrate type [Artavanis-Tsakonas et al., 1999]. BMP is a member of the TGFβ superfamily of ligands and can elicit a large variety of cellular responses. BMP signaling shares some principle features with Notch signaling, particularly the transmission of the signal from the exterior of the cell that involves only a few intermediates and requires the relocation of a signaling component from the cytoplasm to the nucleus [Attisano and Wrana, 2002]. Present study examines the inductive effect of simulated microgravity on differentiation of BMOL stem cells and elucidates the molecular mechanisms underlying the differentiation of BMOL cells under simulated microgravity treatment.

MATERIALS AND METHODS

MATERIALS

Williams' E medium, insulin, transferin, selenium (ITS) premix were purchased from PAN Biotech (Aidenbach, Bavaria). Fetal bovine serum (FBS) was from Invitrogen Life Technologies. Dorsomorphin (DM), propidium iodide (PI), trypan blue was purchased from Sigma Chemical Co. (St. Louis, MO). Annexin V-FITC apoptosis detection kit was purchased from Calbiochem, EMD Chemicals, Inc. (Darmstadt, Germany). 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyl-tetrazolium bromide (MTT), dexamethasone was purchased from Hi-Media (India). Polyclonal antibody to HNF4-α and monoclonal antibody to β-actin were purchased from Santa Cruz. BMP4, Notch1, c-Myc antibodies and recombinant BMP4 were purchased from Abcam. All other chemicals were of the reagent grade and were obtained commercially.

CELL CULTURE AND DIFFERENTIATION OF STEM CELLS

BMOL cells were obtained form Prof. George Yeoh, University of Western Australia, Perth, Australia [Tirnitz-Parker et al., 2007] and maintained in Williams' E medium supplemented with glutamine, 10% FBS, 1% penicillin and streptomycin and referred as normal media. Cells were either grown to high density for 20 days in normal media or the medium was changed after 10 days to Williams' E containing 10% FBS, antibiotics, ITS + Premix (6.25 μg/ml each), 10 mM nicotinamide and 10^{-7} M dexamethasone (referred to as differentiation medium) and kept for another 10 days. Cells grown under normal proliferating conditions to 90% confluency served as a control [Tirnitz-Parker et al., 2007].

SIMULATION OF MICROGRAVITY CONDITIONS

Simulated microgravity was induced using indigenously fabricated 3D clinostat machine as described earlier by our group [Siamwala et al., 2010a,b].

The formula for microgravity (g′) is

$$g\prime = \omega^2 R/g \text{ where } g = 9.8\ m/s^2$$

where ω is the angular velocity in rad/s, R is the radius from the center of rotation, the random positioning machine (RPM) rotates with a constant angular velocity (ω) where angular velocity is equal to angular displacement in radians/time taken is $\theta/\tau = 2$ rads/s.

The sample holders were mounted on the center of the platform located on the inner frame and can experience 10^{-3} gravity. Controls samples were kept in customized vertical shaker to provide movements. However the movements were not randomized as in case of RPM and this set was referred as gravity treated cells. To avoid any nonspecific effect due to treatment conditions, both vertical shaker and RPM machine were kept in a glass chamber maintaining temperature at 37°C and relative humidity.

TREATMENT AND INCUBATION

Cells were grown initially for 10 days in normal media and treated under microgravity for 2 h followed by incubation with normal and differentiation media, respectively. During treatment, the 12-well plates were completely filled with media to avoid the presence of air bubble and sealed with thin UV sterilized plastic cover with thick sheet of filter paper to make sealing tight thereby blocking the leakage of media. Similarly, to avoid any nonspecific effect due to culture conditions, the control 12-well plates were filled with media followed by sealing as mentioned above and treated in vertical shaker and served as movement control.

MEASURING APOPTOSIS USING PI INCORPORATION ASSAY

PI has been widely used to measure apoptosis in different experimental systems. The experiment was performed as stated elsewhere [Srinivas et al., 2003; Majumder et al., 2009]. BMOL cells were seeded in 12-well plate and incubated for 24 h and were treated under simulated microgravity for 2 h while gravity treated cells maintained under same culture conditions were served as control.

ANNEXIN V MEASUREMENT USING ANNEXIN V-FITC APOPTOSIS DETECTION KIT

BMOL cells after 24 h of seeding were subjected to 2 h simulated microgravity and control gravity treatments. After incubating with normal media, the treated cells were processed using the protocol as supplied by the manufacturer (Merck, Calbiochem, EMD Chemicals, Inc.). Fluorescence images of the cells were taken and the numbers of annexin V-FITC-positive cells were counted per field.

CELL VIABILITY ASSAY USING TRYPAN BLUE

Cell viability was measured following the protocol as mentioned earlier [Majumder et al., 2008]. In brief, BMOL cells were seeded in 12-well plates and incubated for 24 h at 37°C/5%CO_2. After incubation, cells were treated under simulated microgravity for 0, 30, 60, 120, and 240 min, respectively. Next, media containing trypan blue (0.4 mg/ml) was added to the cells and incubated for another 15 min. After incubation, the media were removed and PBS was added to all the wells. Random images were acquired using Nikon Cool Pix camera adapted to a Motic inverted microscope and the numbers of cells having a blue nucleus were counted from the images.

CELL TRACKER ASSAY TO DETECT LIVE CELLS

Cell viability was measured using 5-chloromethylfluorescein diacetate probe (CMFDA). BMOL cells were subjected to 2 h simulated microgravity and control gravity treatments. After 24 h incubation with normal media, cells were probed with CMFDA and incubated further for 2 h. Next, cells were washed with PBS and images were taken using Olympus IX71 microscope adapted with DP71 camera.

PROLIFERATION ASSAYS

MTT assay. Cell proliferation was assessed using MTT assay, which allows the quantification of viable cells. BMOL cells were grown in 12-well plates for 24 h and were subjected to 2 h simulated microgravity and control gravity treatments, and incubated for next 24 h in normal media. During the experiment cells were maintained in undifferentiated conditions. MTT (0.2 mg/ml) was then added to media and incubated for 2 h. At the end of the incubation, medium was removed and the formed dye was solubilized with isopropanol. Absorbance of converted dye was measured at a wavelength of 570 nm in a Varian Cary 4000 UV–Vis spectrophotometer.

HEMOCYTOMETER COUNTING

The proliferation status of BMOL cells during microgravity treatment was screened by hemocytometer counting. Twenty-four hours after plating, BMOL cells were subjected to 2 h simulated microgravity treatments. Next, the cells were incubated for the next 24 h in normal media, and subjected to trypsinization to prepare 1 ml of cell suspension in normal media. During the experiment, cells were maintained in undifferentiated conditions. Cell suspension was loaded in a hemocytometer and the numbers of cells were counted under Motic inverted microscope.

TRANSFECTION AND PREPARATION OF CONDITIONED MEDIA (CM)

BMOL cells were grown on 30 mm tissue culture dishes to 70% confluence. Next, the cells were serum starved for overnight and incubated for 8 h with lipofectin reagent containing chordin plasmid. The media were replaced with fresh media containing 10% FBS. After 36 h of transfection, fresh media were added to the dish and incubated for 12 h. Next, the media were taken out and served as chordin containing CM.

IMMUNOFLUORESCENCE ANALYSIS

BMOL cells were plated in 12-well plates and incubated for 10 days to reach 90% confluence followed by microgravity treatment for 2 h. After treatment, cells were incubated for another 2 days and immunofluorescence studies were carried out using the cold paraformaldehyde–Triton X-100 procedure as stated earlier [Majumder et al., 2009]. Cells were incubated at 4°C overnight with mouse monoclonal antibodies (dilution 1:1,000) against HNF4-α. Next, corresponding goat anti-mouse secondary antibodies (dilution 1:2,000) tagged with FITC was used. Images were collected using an Olympus IX71 epifluorescence microscopy system equipped with a DP71 camera.

WESTERN BLOT ANALYSIS

Various sets of BMOL cells of 90% confluence with 10 days incubation in a 12-well plate, on prior to 2 h simulated microgravity and control gravity treatments, were fed with normal and differentiation media for 10 days which were harvested in every consecutive days (i.e., at 0th, 2nd, 4th, 6th, 8th, and 10th day of incubation) for Western blot analysis [Laemmli, 1970]. Total protein concentration was normalized using Biuret Assay and UV absorption of the protein samples. Proteins were detected using 1:500 dilution of HNF4-α antibody (H79, Santa Cruz), Notch1 (ab27526, Abcam), BMP4 (ab39973, Abcam), cMyc (ab32, Abcam) antibodies and 1:1,000 dilutions of goat anti-rabbit IgG conjugated to horseradish peroxidase (Banglore GENEI). The blots were developed using substrate TMB/H_2O_2. Loading control has been performed using the β-actin house keeping gene expression level.

LACTATE DEHYDROGENASE (LDH) ACTIVITY AND UREA PRODUCTION

After 2 days of simulated microgravity treatment, the LDH enzyme activity and production of urea was measured in microgravity treated BMOL cells under all culture conditions described above. Production of urea was measured by modified Berthelot method. The culture media from control gravity treated and microgravity treated cells were used to quantify secreted urea level in the media. Cells were lysed with 50 mM Tris–Cl buffer pH 7.2 containing protease inhibitor cocktail (Sigma Chemicals Co.). The cell lysate from the above-mentioned cells of same culture conditions were prepared and LDH activity was measured by following IFCC/UV Kinetic Lactate to Pyruvate assay protocol.

STATISTICAL ANALYSIS

All the experiments were performed in triplicate ($n = 3$) unless otherwise specified. Data are presented as mean + SE. Data were analyzed using the one-way ANOVA test, the Student's *t*-test and the Tukey post hoc test, as appropriate. *P* values smaller or equal to 0.05 were used as the criterion for a statistically significant difference.

RESULTS

PROLONGED MICROGRAVITY TREATMENT FOR MORE THAN 2 H AUGMENTED CELLULAR DEATH

BMOL cells were treated under different time dosage of microgravity to evaluate the effect of microgravity treatment on cellular health. BMOL cells plated in 24-well tissue culture dishes were subjected to microgravity for 0, 30, 60, 120, and 240 min and evaluated for their viability status using trypan blue staining protocol. Counting of the blue colored nucleus containing cells elaborated a 2.3-fold increase in cell death after 240 min of microgravity treatment (Fig. 1B). However, microgravity treatments for 30, 60, and 120 min did not induce cell death (Fig. 1B). Based on this observation, treatment time of 120 min (2 h) was employed for rest of the experiments.

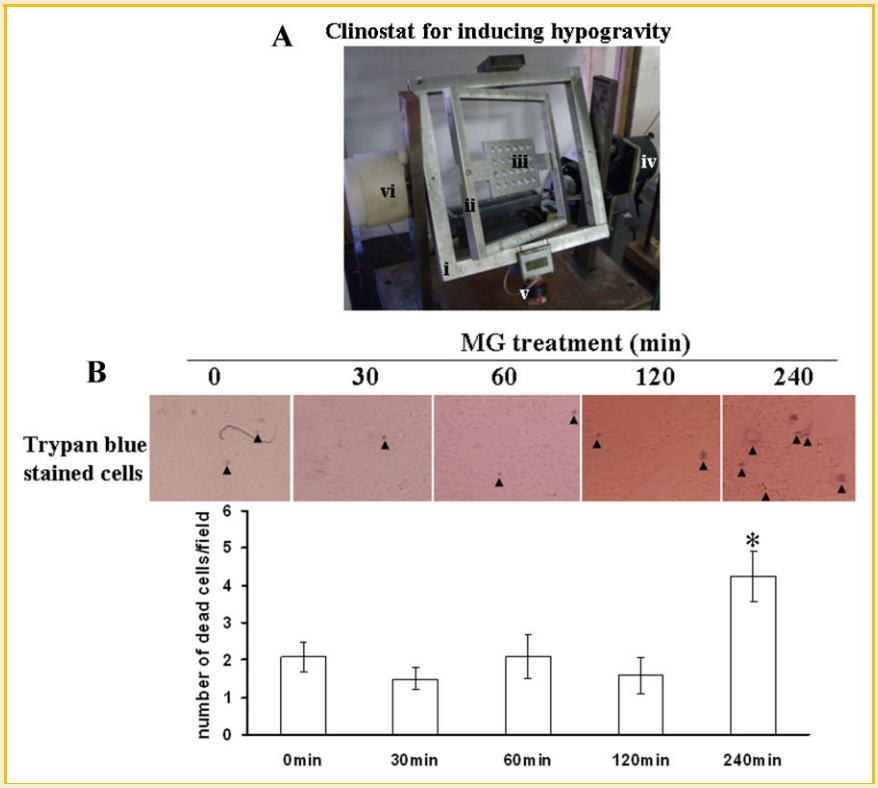

Fig. 1. Clinostat to simulate microgravity and standardizing the time of treatment. A: Clinostat for inducing microgravity. Outer frame (i) which rotates unidirectionally by 20 rpm and an Inner frame (ii) having sample holder (iii) which rotates bidirectionally by 10 rpm. Motor (iv) that rotates the outer frame and Motor (v) that rotates the inner frame and its rotational direction and velocity is being controlled by the controller (vi) connected to a computer having clinostat control program. B: BMOL cells of 90% confluence with 10-day incubation in a 12-well plate were treated in 3D clinostat for microgravity with different time durations include 0, 30, 60, 120, and 240 min and evaluated for their viability status using trypan blue staining protocol (A). Indication of blue colored cells is the fact of cell death, and counting of the blue colored nucleus stained cells elaborated a 2.3-fold increase in cell death after 240 min of microgravity treatment than other durations. $^{*}P < 0.05$ versus 0 min MG treated cells ($n = 5$).

MICROGRAVITY INDUCED CELL PROLIFERATION WITHOUT INTERFERING WITH CELL APOPTOSIS

BMOL cells were treated under microgravity for 2 h followed by probing with annexin V-FITC to check apoptotic cells. Quantification of green fluorescing cells from each field revealed no change in apoptotic status of the cells under microgravity treatment (Fig. 2A). Further, microgravity treated cells were probed with PI to locate the late apoptotic cells. No significant difference in number of late apoptotic cells was observed between gravity and microgravity treatments (Fig. 2B). Microgravity treated cells were also probed with cell tracker, a fluorescent probe for the detection of viable cells (Fig. 2C). The data confirm that 2 h microgravity treatment is not lethal to the cells.

Microgravity treated cells were studied for the proliferation status using MTT assay and hemocytometer counting technique. Hemocytometer counting of cells depicted that microgravity treatment augmented cellular proliferation by 1.74-fold (Fig. 2D) while MTT assay showed 1.5-fold increase in cell proliferation under microgravity treatment (Fig. 2E). Overall, these sets of data elucidated that 2 h microgravity treatment augmented cellular proliferation without inducing apoptosis and compromising cell viability.

MICROGRAVITY INDUCED THE DIFFERENTIATION OF BMOL CELLS

BMOL cells were plated in 12-well plates and maintained for 10 days unless it reaches 90% confluence. Next, the cells were treated under microgravity for 2 h followed by incubation with normal and differentiation media for the next 10 days. Samples from the treated cells were prepared from each day of incubation among different sets and probed for HNF4-α expression, a marker of hepatocytes. Samples from 0 day microgravity treatment showed absence of HNF4-α expression thus demonstrating the absence of differentiated cells (Fig. 3A). After 2 days of microgravity treatment, the cells showed 1.3-fold higher level of HNF4-α expression with normal media while microgravity treated cells incubated with differentiation media showed 2.9-fold more HNF4-α expression compared to gravity controls (Fig. 3B). Microgravity treated cells incubated with differentiation media depicted higher level of HNF4-α expression than all other sets at 4th day of incubation (Fig. 3C). From 6th day onwards variable level of HNF4-α expression was observed in different sets (Fig. 3D). However, low level of HNF4-α expression was observed under gravity treated cells incubated with normal media compare to all other treatments (Fig. 3E,F).

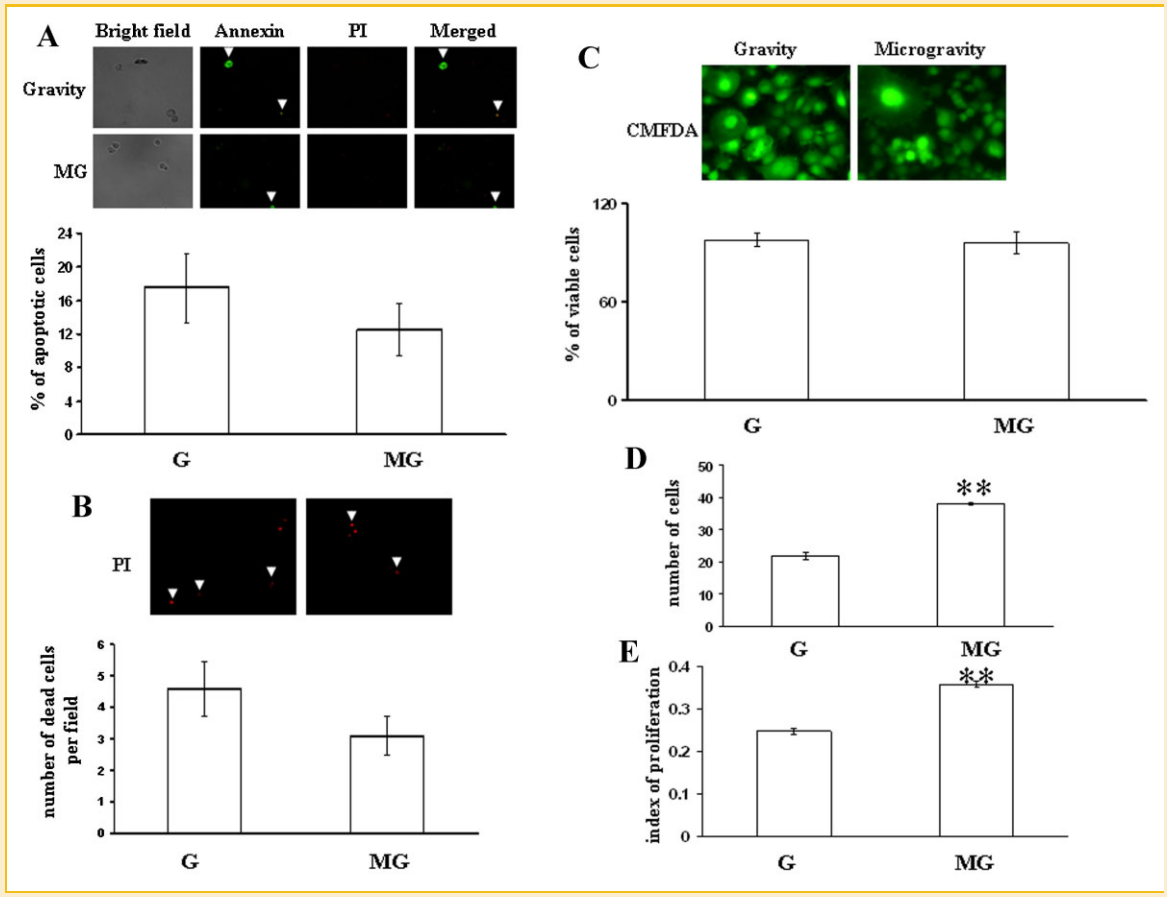

Fig. 2. Microgravity treatment for 2 h induced proliferation of stem cells without inducing apoptosis. The level of apoptosis during microgravity treatment was detected by an annexin V-FITC/PI kit. Green fluorescence by Annexin-V, and red fluorescence by PI were imaged under microscope along with bright field images. The number of green fluoresced cells as shown by white arrows, which are the index of apoptosis, were counted and plotted as percentage of apoptotic cells. No significant difference in number of late apoptotic cells was observed between gravity and microgravity treatment (A) ($n = 3$). The index of apoptosis during microgravity treatment was assessed by PI assay. Once the live cells were stained with PI, Bright field and fluorescence images were taken under microscope. Number of PI stained cells as shown by white arrows were counted and plotted (B) ($n = 3$). The number of viable cells under microgravity treatment was measured with CMFDA. CMFDA binds to reduced glutathione and gives green fluorescence, which was imaged under microscope. Images showed no cell tracker unstained nonviable cells under microgravity (C) ($n = 3$). The proliferation status of BMOL cells during microgravity treatment was screened by hemocytometer counting. Cells were treated under microgravity and incubated overnight with normal media. Number of cells were counted using hemocytometer and plotted. Microgravity treatment augmented cellular proliferation (D). $^{**}P < 0.001$ versus gravity ($n = 5$). The result of hemocytometer counting was further confirmed by MTT assay, which measures the viability of cells. The yellow colored oxidized MTT was reduced to colorimetrically measurable purple colored formazan by mitochondrial reductases only from the viable cells (E). $^{**}P < 0.001$ versus gravity ($n = 5$).

Microgravity treatment without differentiation media induced differentiation by 31% while microgravity treatment with differentiation media by 23% than gravity control respectively (Fig. 4A). Gravity treated cells incubated with differentiation media showed 37% higher differentiation compare to gravity control cells incubated with normal media (Fig. 4A). Morphology counting of differentiated cells also revealed higher differentiation under microgravity treatment (Fig. 4B). Urea secretion [Herrera et al., 2006] and LDH activity [Ghosh et al., 1995] was also taken as hepatocyte markers to study the differentiations. Microgravity treated cells elaborated higher level of urea secretion after 2 days of microgravity treatment (Fig. 4C). The results of LDH activity measurement assay revealed higher LDH activity in microgravity treated cells incubated with differentiation media (Fig. 4D).

MECHANISM BEHIND MICROGRAVITY DRIVEN DIFFERENTIATION OF STEM CELLS

The onset of stem cell differentiation was observed on 2nd day after 2 h microgravity treatment. Therefore, we selected 2 days incubated microgravity treated cells to dissect out the underlining mechanism of stem cells differentiation.

Notch and BMP signaling are highly implicated in differentiation of stem cells [Xu et al., 2002; Androutsellis-Theotokis et al., 2006; Chiba, 2006; Nostro et al., 2008; Yu et al., 2008]. We carried out one positive control experiment where the cells were gone through normal differentiation process as mentioned earlier and evaluated for their Notch1 and BMP4 expression profile. No work has been reported yet describing the role of BMP4 and Notch1 signaling in differentiation of BMOL cells. In this set of study, we performed experiments to confirm the role of BMP4 and Notch1 signaling in

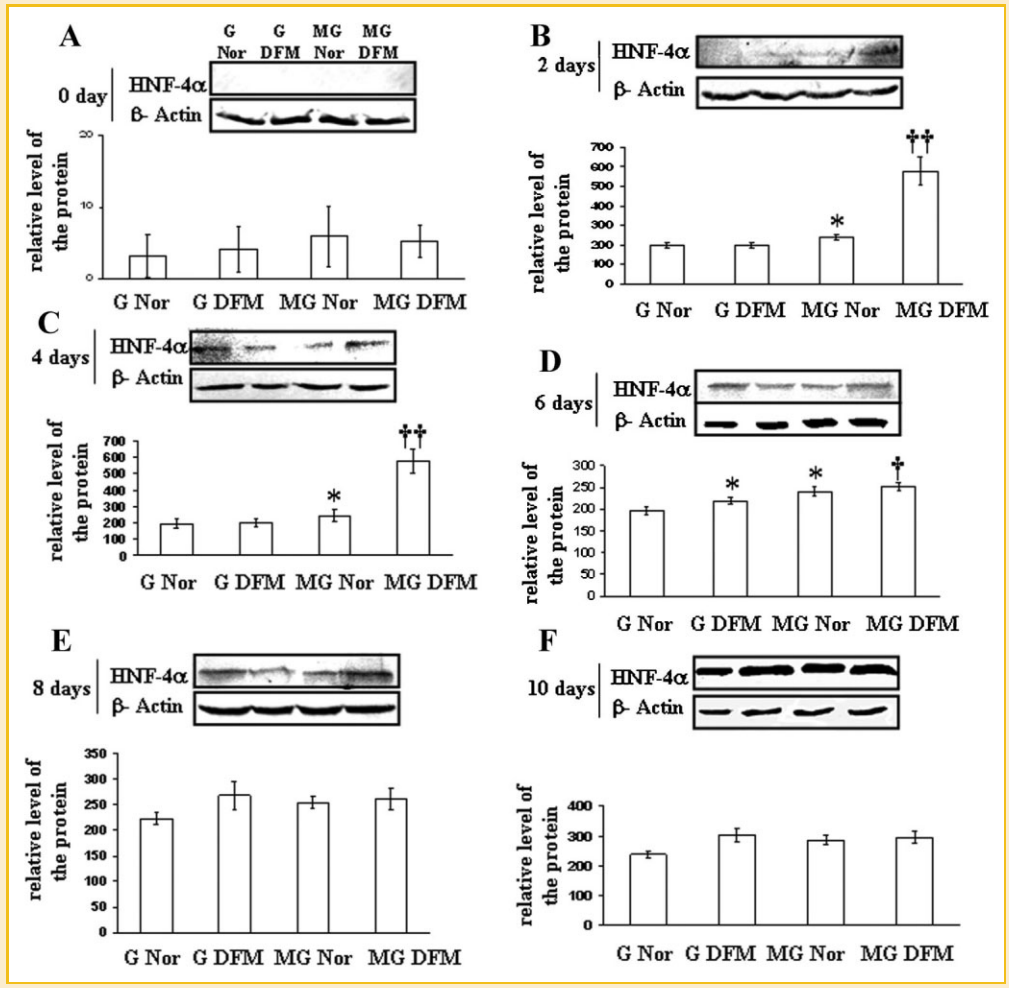

Fig. 3. A–F: Day dependent microgravity inductive expression of hepatocyte marker (HNF4-α) in BMOL cells. Western blot analysis for the expression patterns of HNF4-α by different sets of 2 h microgravity treated BMOL cells fed with normal and differentiation media for various days from day 0 to day 10. The densitometry quantification of band intensities indicate the relative expression of HNF4-α in BMOL cells. * and ** versus gravity normal ($^{*}P < 0.05$; $^{**}P < 0.001$), † and †† versus gravity DFM (n = 3).

differentiation of BMOL cells. Differentiated cells were confirmed by HNF4-α expression and also showed 2.2-fold higher level of BMP4 expression than undifferentiated cells while differentiated cells expressed 2.4-fold less Notch1 than undifferentiated cells (Fig. 5A).

Next, microgravity treated cells were probed for BMP4 and Notch1 expression to evaluate the effect of microgravity on these two signaling molecules. Cells were treated under microgravity for 2 h followed by incubation for 0, 2, and 10 days. Probing BMP4 and Notch1 in 0 day incubated sample elucidated that Notch1 is equally express in microgravity and gravity treated cells incubated with and without differentiation media while 1.55-fold more BMP4 was expressed in microgravity treated cells than gravity treated cells when compared between the respective sets (Fig. 5B). These data elaborated that microgravity treatments switched on and upregulated the expression BMP4 to induce differentiation of the cells.

Probing BMP4 and Notch1 in 2 days incubated microgravity treated cells demonstrated a 2.2- and 1.3-fold increases in BMP4 expression level in microgravity treated cells incubated with and without differentiation media, respectively, while 3.2- and 1.7-fold reductions in Notch1 expression level were observed in microgravity treated cells incubated with and without differentiation media, respectively (Fig. 5C).

Further, samples from 2 h microgravity treated and 10 days incubated cells were carried out in SDS–PAGE system and probed for BMP4 and Notch1. Densitometry analysis of the band elaborated the presence of high level of BMP4 in microgravity treated cells incubated with differentiation media while higher level of Notch1 expression was observed in gravity control cells incubated with normal media (Fig. 5D). The lowest BMP4 expression level was observed in gravity treated cells incubated with normal media while the lowest Notch1 expression level was measured in microgravity treated cells incubated with differentiation media (Fig. 5D). These sets of data suggested that microgravity upregulated BMP4 signaling and downregulated Notch1 signaling which may play crucial role in microgravity driven differentiation of these stem cells.

BLOCKING BMP4 SIGNALING BLUNTED MICROGRAVITY DRIVEN DIFFERENTIATION OF BMOL CELLS

Stem cells were transfected with chordin plasmid following lipofectine protocol as mentioned in materials and methods. After

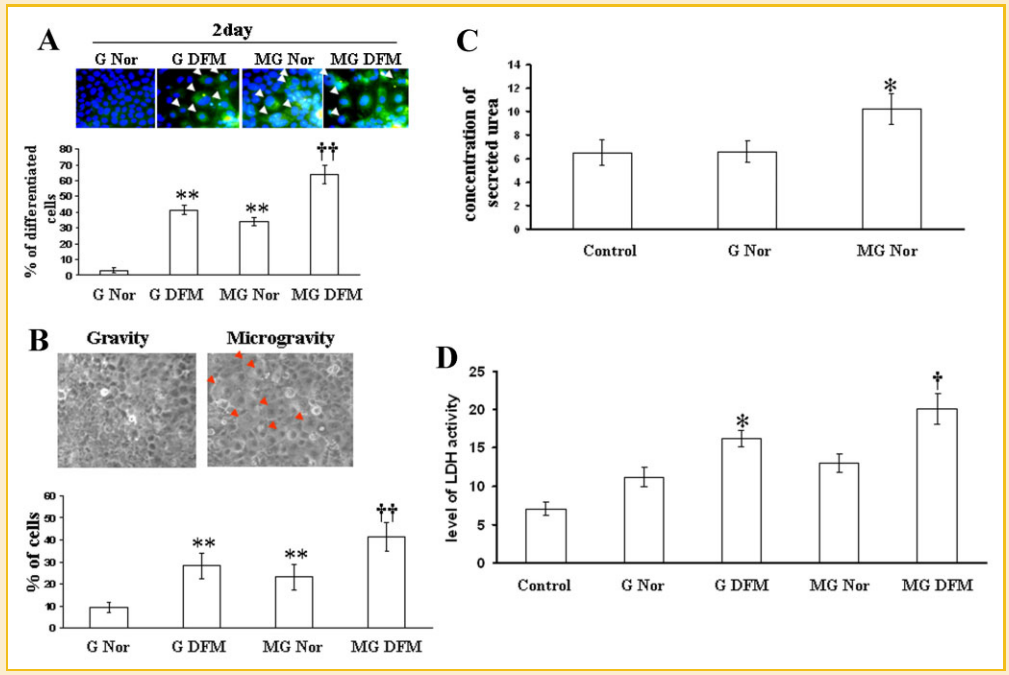

Fig. 4. Microgravity induced BMOL differentiation. A: The number of FITC-positive cells as shown by white arrows originated from the bindings of antibodies to HNF4-α was counted and plotted. B: Differentiated cells identified by their morphology from gravity and microgravity treated sets were counted and plotted. C: Urea in 12 h incubated culture media were measured using Berthelot method and plotted (n = 3). D: Treated cells were lysed with 50 mM Tris–Cl buffer pH 7.2 containing protease inhibitor cocktail (Sigma Chemicals Co.). LDH activity was measured in the cell lysate using IFCC/UV Kinetic Lactate to Pyruvate assay protocol (n = 3). * and ** versus gravity normal (*$P < 0.05$, **$P < 0.001$), † and †† versus gravity DFM (†$P < 0.05$, ††$P < 0.001$).

48 h incubation, CM from the transfected cells were collected and used as chordin CM for treating the cells. Expression of chordin was detected using c-Myc expression in CM (Fig. 6A).

A positive control experiment was performed where the cells were grown for initial 10 days in normal media to reach 90% confluence followed by incubation with differentiation media for another 10 days. In another set of treatment, cells were initially incubated with normal media for 10 days followed by incubation with chordin containing differentiation media. Probing HNF4-α in the cells elaborated that chordin containing differentiation media blocked the differentiation of BMOL cells by 4.7-fold thus elaborating the importance of BMP4 signaling in differentiation of BMOL cells (Fig. 6B).

Further, microgravity treated cells were incubated for 2 days with differentiation media with and without chordin. The microgravity treated cells which are incubated with normal differentiation media showed 12.5-fold more HNF4-α expression than those incubated with chordin containing differentiation media (Fig. 6C). In parallel, another set of experiments were performed where the cells were treated under microgravity and incubated with normal media with and without chordin. Microgravity treated cells that incubated with normal media elucidated a 4.7-fold high level of HNF4-α expression than the cells incubated with chordin containing normal media (Fig. 6D). We further used DM, a selective inhibitor of BMP and also recombinant BMP4 and tried to study the effect of these two on BMOL cells differentiation under simulated microgravity. We found that DM blocked simulated microgravity driven differentiation of BMOL cells while administration of recombinant BMP4 synergistically induced simulated microgravity driven differentiation of BMOL cells (Fig. 6E). These sets of data elaborated that high level of BMP4 expression after microgravity treatment is a key modulator behind microgravity driven differentiation of stem cells.

DISCUSSION

Stem cells have long been used as therapeutic modalities in different disease conditions. Faster differentiation of liver stem cells to mature hepatocyte is required to regenerate the injured liver. The present study describes microgravity as a potential physical force, which can enhance the proliferation and differentiation of liver stem cells. We also identified that Notch1 and BMP4 are the key modulators that play an important role in microgravity-dependent differentiation. We also elaborated that BMP4 being a crucial molecule in development plays important role in BMOL cell differentiation under both gravity and microgravity conditions, and thereby acting as an important hub in regulating differentiation of BMOL cells under both gravity and microgravity conditions.

In recent years, microgravity has been shown as a physical force playing differential roles in proliferation and differentiation of various stem cells [Yuge et al., 2006; Dai et al., 2007; Gershovich and Buravkova, 2007; Li et al., 2009]. Findings of Li et al. [2009] reported enhanced proliferation and differentiation of human periodontal ligament stem cells under microgravity [Gershovich and Buravkova, 2007] while Dai et al. [2007] showed inhibitory effect of simulated microgravity on proliferation and differentiation of rat bone

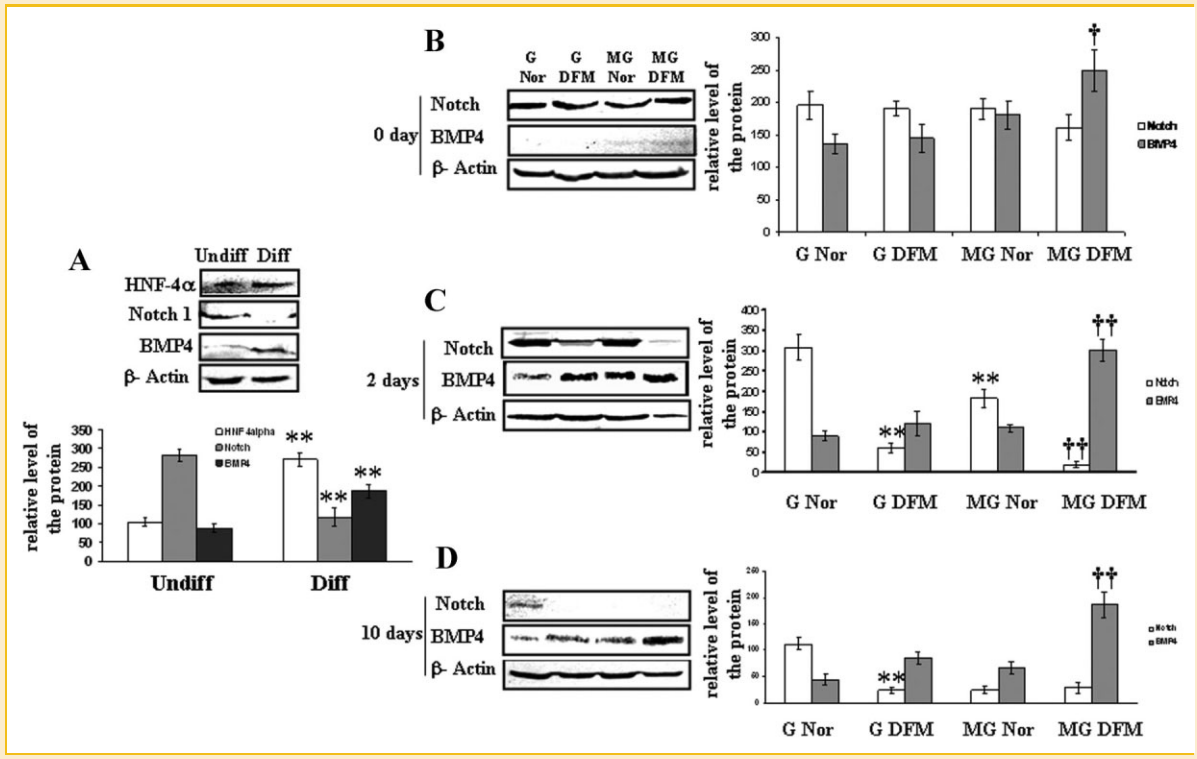

Fig. 5. Role of BMP4 and Notch1 signaling in microgravity driven differentiation of BMOL cells. A: Western blot analysis of Notch1, BMP4, HNF4-α and β-actin was carried out on BMOL cells went through normal differentiation process. ** versus undiff. (**$P < 0.001$). B–D: Western blot analysis was carried out on various sets of 10 days incubated BMOL cells subjected to 2 h microgravity treatment followed by incubation with normal and differentiation media for 0, 2, and 10 days. The samples were probed with Notch1, BMP4, and β-actin and the band intensities were estimated using densitometry quantification module of Image J. ** versus gravity normal (**$P < 0.001$), †† versus gravity DFM (††$P < 0.001$) ($n = 3$).

marrow mesenchymal stem cells. Earlier reports also depicted that microgravity induced the proliferation status of human mesenchymal stem cells without changing their differentiating property [Yuge et al., 2006]. Therefore, these reports suggested that stem cells from different origin respond differentially under microgravity condition. Previous findings reported that longer time treatment under microgravity could induce apoptosis of the cells thus compromising the viability of the cells [Monici et al., 2006; Liu and Wang, 2008]. Viability study of BMOL cells treated at different time points under microgravity, revealed that 4 h and more of microgravity treatment partly compromised cellular viability. However, no change in cellular apoptosis and cell viability was observed in 2 h microgravity treated BMOL cells. On the basis of this observation, we treated cells under microgravity for 2 h.

Herrera et al. [2006] reported that microgravity induced the differentiation of human liver stem cells to mature hepatocyte thus expressing cytochrome P450, and albumin, which are possible markers of differentiated stem cells. BMOL cells are known to differentiate into cholangiocytes and hepatocyte according to treatment conditions [Tanimizu and Miyajima, 2004]. Mature cholangiocytes strongly express HNF1-β, but barely express HNF4-α while HNF4-α is more predominantly expressed than HNF1-β in mature hepatocyte [Tanimizu and Miyajima, 2004]. In the present study, we observed that short-time microgravity treatment for 2 h induced the differentiation of oval cells to mature hepatocyte. Maximum difference in HNF4-α expression was observed after 2 days of post-microgravity treatment. Microgravity alone induced the differentiation of stem cells without biochemical modification using the differentiation media. Additionally, microgravity facilitated the process of differentiation thus reducing the total time required by BMOL cells to differentiate. However, longer incubation for more than 5 days of post-microgravity treatment showed an equal level of HNF4-α expression elaborating that maximum portion of the stem cell population have already differentiated to mature hepatocytes thus expressing almost equal level of HNF4-α. We also measured the urea secretion and LDH activity in microgravity treated cells and detected a higher urea secretion and LDH activity in cells treated under microgravity.

BMP is a member of the TGFβ superfamily of ligands and can elicit a large variety of cellular responses. It is also reported that BMP and Fibroblast growth factor (FGF) signaling are involved in hepatic differentiation [Jung et al., 1999; Rossi et al., 2001]. Shiraki et al. [2008] reported that addition of noggin, a BMP4 antagonist abolished the hepatic differentiation of mouse and human embryonic stem cells, which was further recovered by FGF addition [Shiraki et al., 2008]. These findings depicted that BMP signaling is required in the induction of hepatocyte differentiation while FGF signaling potentiates BMP signal. Earlier studies reported that in vitro generation of embryonic stem cell-derived hepatic cells using BMP4 [Gouon-Evans et al., 2006]. Additionally, findings suggested

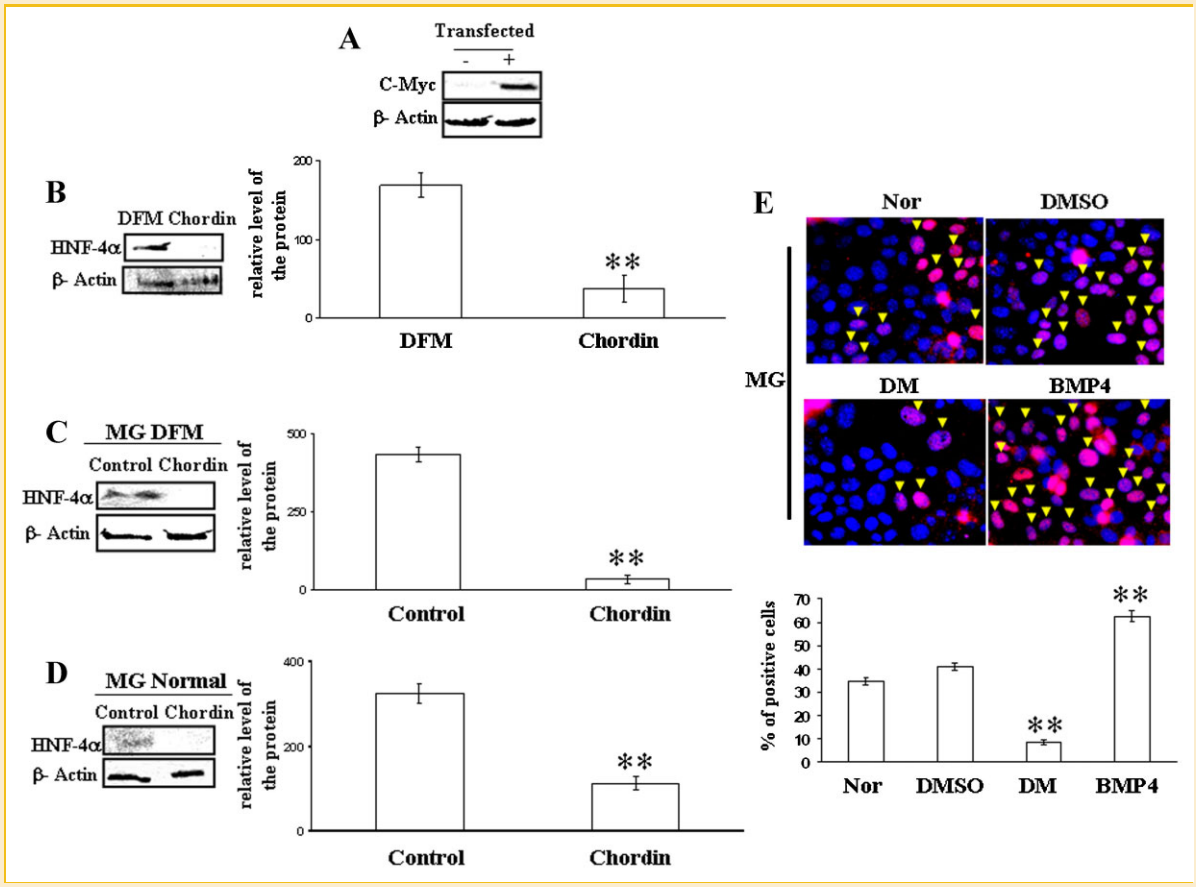

Fig. 6. Blocking BMP4 signaling blunted microgravity driven differentiation of BMOL cells. A: Chordin transfection was confirmed by the Western blot analysis by probing with c-Myc. B: Western blot analysis was carried out on BMOL cells of 90% confluence with 20 days incubation in a 12-well plate, on which the normal media were replaced with differentiation media with and without chordin after 10th day, which were probed for HNF4-α. ** versus DFM (**$P < 0.001$). C: Western blot analysis was carried out on 10 days incubated BMOL cells treated under microgravity for 2 h were fed with differentiation media with and without chordin for 2 days, which were probed for HNF4-α (D) the same was carried out with normal media with and without chordin. ** versus control (**$P < 0.001$). E: After growing BMOL cells in normal media for 10 days, cells were treated under microgravity for 2 h followed by incubation with and with out DM and BMP4, respectively. Treatment with DM and BMP4 were given both 0th and 1st day of incubation after microgravity treatment. At 48th hour of incubation, cells were then processed for immunofluorescence and probed for HNF4-α. Presence of pink colored nucleus due to the co-localization of DAPI and TRITC representing the presence of HNF4-α demonstrated the presence of differentiated hepatocyte from the population. Number of pink nucleus per field were counted and plotted as percentage of positive cells. ** versus respective controls (**$P < 0.001$).

that BMP4 stimulated expression of transcription factor—C/EBP-alpha, which involved in differentiation of WB-F344 cells toward hepatocytes lineage [Fan et al., 2009]. Work of Cai et al. [2007] reported that not only BMP2, treatment with BMP4 can also able to induce the differentiation of human embryonic stem cells to functional hepatic cells. Therefore, as reported by different groups, we selected BMP4 as the target candidate from BMP family to examine its role in microgravity driven differentiation of BMOL cells to hepatocyte. Understanding the importance of BMP signaling in hepatocyte differentiation, we studied BMP4 signaling in microgravity treated BMOL cells and found that microgravity induces the expression of BMP4 in BMOL cells and thereby switches on the autocrine signaling leading to differentiation of BMOL cells to mature hepatocytes. Additionally, we reported for the first time that differentiation of BMOL cells to mature hepatocytes involves BMP4 signaling, and interplaying with it can inhibit the differentiation of BMOL cells to mature hepatocyte. We used DM and chordin, a BMP4 antagonist and showed that blocking BMP4 signaling in microgravity treated BMOL cells can attenuate the microgravity driven differentiation of stem cells. Thus, our findings demonstrated the importance of BMP4 signaling in controlling differentiation of BMOL cells to mature hepatocytes under both gravity and microgravity conditions.

Transmission of signals due to cell–cell interaction through Notch receptor leads to relocation of certain signal components such as BMP from cytoplasm to nucleus [Gaiano and Fishell, 2002; Dontu et al., 2004]. Frequently, increased activation of Notch1 exhibits immortalized state of stem cells with downregulation of BMP4 signals. Decrease in the expression of Notch1 ameliorates the expression of BMP4, which activates various transcription factors that are involved in the regulation of stem cell differentiating genes. Notch has attracted the attention of researcher working with various stem cell systems of higher vertebrates due to the differentiation inhibitory activities of Notch signaling which is physiologically important for maintaining the undifferentiated state of stem cells [Artavanis-Tsakonas et al., 1999; Molofsky et al., 2004; Suzuki and

Chiba, 2005]. However, Notch regulation occurs in very differential way during differentiation of stem cells. Cholangiocytes strongly express HNF1-β, but barely express HNF1-α, HNF4, and C/EBP-α, activation of the Notch signaling upregulated HNF1-β expression, whereas it downregulated the expression of HNF1-α, HNF4, and C/EBP-α. However, during differentiation of stem cells to mature hepatocyte HNF4-α, a potential marker of hepatocyte gets expressed. These studies suggested that the Notch signaling contributes to form a network of these transcription factors and control the differentiation of stem cells [Tanimizu and Miyajima, 2004]. Nishikawa et al. [2005] elaborated that Notch1 signaling is important in hepatocyte differentiation and predominant than Notch2 and Notch3 [Nishikawa et al., 2005]. Therefore, Notch signaling is implicated in regulation of liver stem cells differentiation to form hepatocyte. Our findings also revealed that microgravity blocked Notch1 expression in cells thus inducing the differentiation of BMOL cells to form hepatocyte. However, no change in Notch1 expression was observed in the samples prepared just after microgravity treatment while 10 days after post-microgravity treatment, only gravity treated cells showed Notch1 expression. These sets of data elaborate that upregulation of Notch1 in BMOL cells help the cells to maintain the undifferentiated state while downregulation of Notch1 potentiate the cells to differentiate to hepatocyte.

Faster growth and differentiation of liver stem cells to mature hepatocyte is one of the key factors during liver regeneration. Lowes et al. [2003] discussed that oval cell like BMOL can be used for liver regeneration after liver injury and also mentioned that once injected into portal circulation; these cells facilitate the process of liver regeneration by differentiating into mature hepatocyte [Lowes et al., 2003]. However, in vitro chemical modification using dexamethasone, various cytokines and growth factors to induce differentiation of liver stem cells takes longer time and cannot be used satisfactorily for clinical purposes.

The present study demonstrated that microgravity can induce the proliferation and differentiation of BMOL cells to mature hepatocyte and thus microgravity activated BMOL cells can be used as therapeutic modalities during liver regeneration after liver injury. Further in vivo and clinical studies using microgravity treated oval cells can reveal the therapeutic potential of microgravity treated stem cells in combating against the diseases like liver fibrosis and early liver injury.

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