Studying the mechanism of transcriptional regulation of stem cell proliferation and differentiation

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Abstract

Mesenchymal stem cells (MSCs) are multipotent adult stem cells which can undergo both self-renewal and multi-lineage differentiation. As an important intracellular second messenger, cAMP has been shown to induce the differentiation of mesenchymal stem cells into neural cells. Elevation of cAMP level is usually tied with the activation of the CREB family proteins, which are important transcription factors involved in proliferation and differentiation. One target gene of the CREB family proteins is cyclin D1. This cell cycle regulator is involved in G1/S phase transition and thus plays a crucial role in proliferation. While activated CREB induces the transcription of cyclin D1, another member of the CREB family protein called ICER inhibits the transcription of cyclin D1 by competing with CREB for binding to the CRE motif. In addition, cyclin D1 has a kB motif in its promoter region, where the transcription factor NF-κB could bind and induce its transcription. NF-κB thereby activates the transcription of cyclin D1 and promotes cell cycle progression. Whether cyclin D1 is up-regulated or down-regulated largely depends on the relative activity of CREB, ICER and NF-κB. Since cyclin D1 is a crucial regulator of G1/S phase transition, its expression may greatly influence cell cycle progression and consequently affect stem cell proliferation versus differentiation.

Introduction

Due to the self-renewal and multi-lineage differentiation ability, stem cells are promising source for treating many diseases. Mesenchymal stem cells (MSCs), which are bone marrow derived adult stem cells, can undergo multi-lineage differentiation and give rise to many other cell lineages such as neuron-like cells under specific experimental conditions (Woodbury 2000, Sanchez-Ramos 2002, Lu 2004). The choice between proliferation and differentiation depends on a number of factors, such as extracellular signals. Extracellular signals are usually transduced through intracellular signaling molecules which may activate transcription factors and regulate the transcription of the target genes. Coordinating these signaling pathways and gene expression regulation is crucial to modulating stem cell fate. Although changes in morphology and expression of marker genes can provide evidence of neural differentiation, it is still poorly understood how stem cells reprogram their gene expression profiles and make decision between proliferation and differentiation.

Researches have shown that increased intracellular cAMP levels would cause MSCs to differentiate into neural cells (Deng 2001, Kim 2005, Chu 2006). Elevated cAMP level usually induces the phosphorylation and activation of the CREB (cAMP response element binding protein) family proteins. It has been shown that activation of CREB parallels neural

differentiation (Murphy 1997, Andreatta 2004, Giachino 2005). Taken together, increased cAMP level typically causes CREB activation, which alters the gene expression profiles and drive the determination between proliferation and differentiation.
Among the CREB target genes, some are cell cycle regulators, such as cyclin D1 and cyclin A (Mayr 2001). Cyclin D1 expression is up-regulated by activated CREB and down-regulated by ICER (inducible cAMP early repressor), the latter is also inducible by activated CREB. Since ICER lacks an activation domain, it can only act as a repressor in regulating cAMP-responsive gene expression (Molina 1993). ICER competes with CREB to bind to CRE motifs and inhibits gene transcription. As a result, the level of cyclin D1 is negatively correlated to the ICER levels. As an important cell cycle regulator, cyclin D1 forms a complex with CDK4 (cyclin dependent kinase 4) or CDK6 to drive the G1/S transition (Hunter 1994). Suppression of cyclin D1 transcription may cause cell cycle arrest and thereby decrease the rate of proliferation.
On the other hand, the transcription factor NF-κB has been reported to inhibit the differentiation of a number of mesenchymal cells. NF-κB down-regulates a critical transcription factor RUNX2 involved in osteoblast differentiation through the TNF-a signaling pathway (Gilbert 2002). NF-κB inhibits the expression of Sox9 and MyoD, transcription factors required for mesenchymal cell differentiate into chondrocyte and myocyte (Sitcheran 2003). Evidence suggests that activation of NF-κB increases the proliferative capacity of neural progenitors (Young 2006, Widera 2006), partly through the regulation of cell cycle progression. Increased NF-κB activity has been shown to up-regulate cyclin D1 activity (Guttridge 1999). Higher NF-κB activity stimulates the transcription of cyclin D1 and thereby promotes cell proliferation.
Therefore, we propose that cAMP facilitates neural differentiation of MSCs by delaying the G1/S phase transition through direct inhibition of the cyclin D1 expression by ICER and indirect inhibition of cyclin D1 expression by down-regulating the NF-κB activity. Down-regulating cyclin D1 expression may disrupt the normal cell cycle progression and drive stem cells towards differentiation.

Experimental procedure

Cell isolation and culture

Bone marrow mesenchymal stem cells are isolated from 6-8 week old Sprague-Dawley female rat as previously described (Hemptinne 2004). Cells are cultured in DMEM (Invitrogen) containing 10% foetal bovine serum (Tissue Culture Biologicals), 100 μg/mL streptomycin (Invitrogen) and 100U/mL penicillin (Invitrogen) and placed in the incubator with a humidified atmosphere containing 5% CO2 at 37oC. Medium is replaced every 3 to 4 days until the cells reach 80% confluence.

In vitro differentiation

Forskolin (Sigma) and IBMX (Sigma) is added to culture medium to reach a final concentration of 10μM and 100μM respectively. Culture medium is changed to medium supplemented with forskolin and IBMX 12 hours after cell seeding and replaced every 3 days until day 6.

Immunohistochemistry

Cells are washed three times with cold PBS and fixed in PBS containing 4% paraformaldehyde for 20 min at room temperature. The cells are rinsed three times with PBS before adding permeabilization buffer (5% normal goat serum (Calbiochem), 0.5% Triton X-100 (Research Products Internationals) in PBS). Permeabilize cells for 30 minutes at room temperature. Wash cells three times with PBS and incubate cells overnight with NF-200 (neurofilament 200) antibody (Sigma) and GFAP (Glial fibrillary acidic protein) antibody (Adv Immunochemical Inc). The cells are then washed with PBS three times and incubated with FITC-conjugated anti-mouse IgG secondary antibody (Chemicon) and Rhodamine-conjugated anti-rabbit IgG secondary antibody (Chemicon). After incubation at room temperature for one hour, the cells are washed and observed under a fluorescence microscope.

MTT assay

Cells were seeded in 96-well plate at density of 10^4 cells/ml. Four hours before the end of treatment, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution is added to each well and the cells are incubated at 37ºC until assay time. At the end of the incubation time, acidic isopropanol is added to dissolve the formazan produced from MTT. The plate is then measured at a wavelength of 570nm with background reading set at 630nm.

LDH assay

Cell cytotoxicity is detected by the cytotoxicity detection kit from Roche. In brief, both released and intracellular LDH (lactate dehrogenase) is collected in cell-free culture supernatant. Substrate is added to the supernantants and reduced by enzymatic reaction. The reduction is captured by the change in color and detected colorimetrically.

Caspase 3 activity assay

Caspase 3 is one of the critical caspases involved in apoptosis. Caspase 3 activity is measured by a kit from BIOMOL. Briefly, Cell extracts were incubated with substrate Ac-DEVD-AMC. The cleavage of the substrate generates fluorochrome and is proportional to the concentration of the activated caspase 3 in cell extracts.

Results

Effect of elevated cAMP on cell proliferation and cytotoxicity

To determine the appropriate concentration of cAMP to induce neural differentiation, different concentrations of forskolin and IBMX were used to treat the rat MSCs (rMSCs) and their levels of LDH release were measured. The release of LDH is an indication of cell damage and cell death. rMSCs treated with forkolin (5μM, 10μM, 20μM and 40μM) combined with IBMX (50μM, 100μM, 200μM and 400μM) showed a protective effect at low concentrations which became cytotoxic when forskolin and IBMX concentration increased (Fig 1). In order to determine whether the elevated cAMP caused cytotoxicity or cell death, caspase 3 activity was measured and was not detectable.

Based upon the cytotoxicity results, we selected the following concentration F10I100 (which contains 10μM forskolin and 100μM IBMX) for the following differentiation experiments.

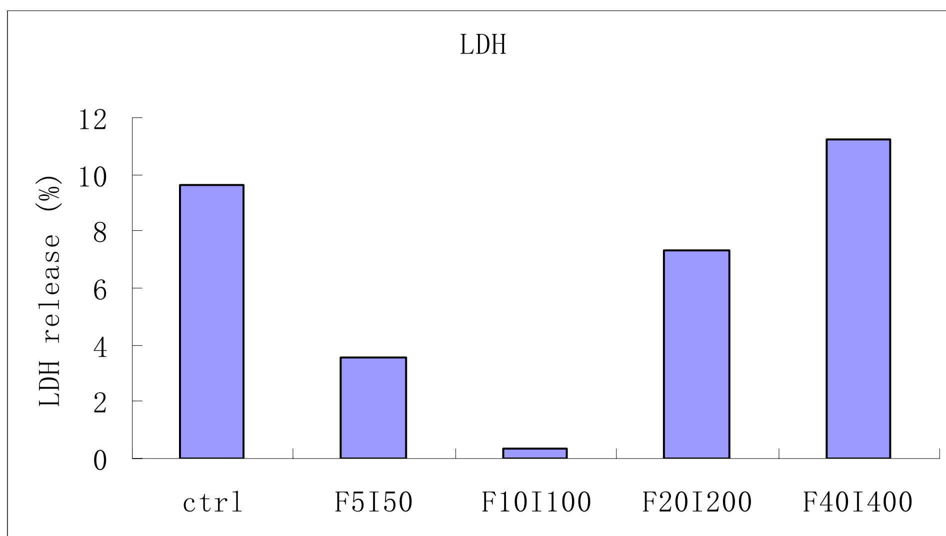
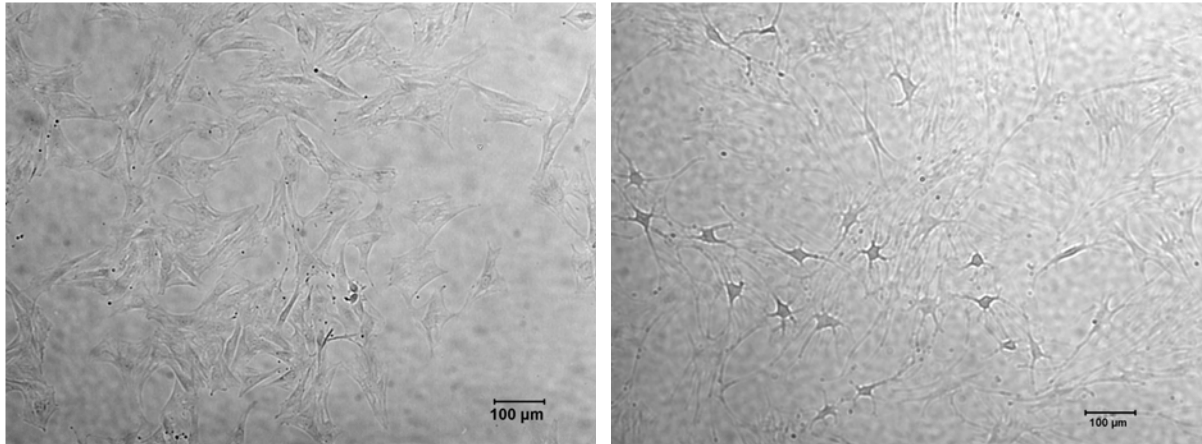


Fig 1 LDH release of rMSCs treated with different concentration of forskolin and IBMX for 6 days. cAMP concentration exerts a protective effect at low concentration and is toxic to rMSCs at high concentrations. (‘F’ stands for forskolin, ‘I’ stands for IBMX, the number following ‘F’ and ‘I’ represent the concentration of forskolin and IBMX, respectively)

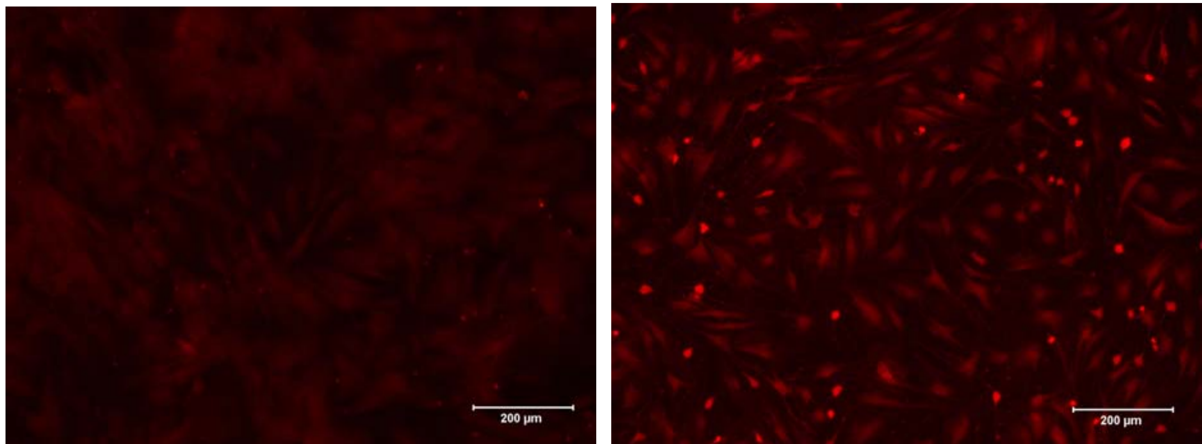
Neural induction of meshenchymal stem cells

rMSCs were cultured in complete medium with 10μM forskolin and 100μM IBMX for 6 days. Morphological changes into the neural cells were apparent (Fig 2). Compared with Fig 2(a), the cells in Fig 2(b) had retracted cytoplasm toward the nucleus and formed multipolar cell body. To determine whether rMSCs differentiated into neural cells, the stem cells were stained for astrocytes using GFAP and for neuron using NF-200. rMSCs showed very little GFAP staining prior to adding forskolin and IBMX, whereas the cells incubated with 10μM forskolin and 100μM IBMX for 6 days exhibited GFAP staining (Fig 3). On the other hand, rMSCs stained for NF-200 both before and after forskolin and IBMX incubation (Fig 4). Tondreau et.al showed that MSCs already expressed certain specific neural proteins before differentiation, which suggests that MSCs retain the ability for neural differentiation (Tondreau 2004).



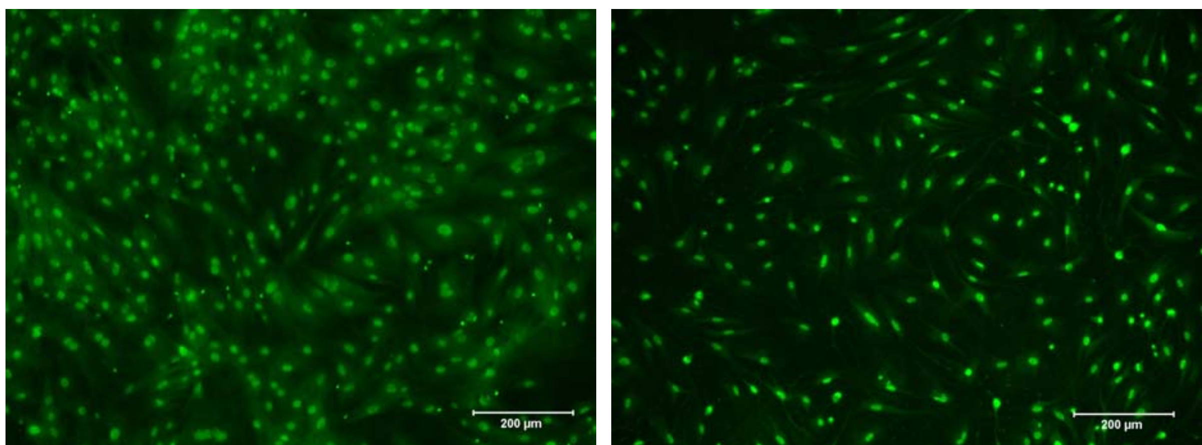
(a) (b)

Fig 2 Morphology of rat MSCs cultured in medium (a) and in medium containing 10µM forskolin and 100µM IBMX (b) for 6 days. Morphological changes into neural cells are apparent. Flat MSCs contract and gain multipolar cell body.



(a) (b)

Fig 3 GFAP expression in Rat MSCs cultured in medium (a) and in medium containing 10µM forskolin and 100µM IBMX (b). After induction, MSCs express GFAP protein, an astrocyte marker.



(a) (b)

Fig 4 NF-200 expression in Rat MSCs cultured in medium (a) and in medium containing 10μM forskolin and 100μM IBMX (b). Cells express NF-200 both before and after differentiation.

Proliferation rate

Stem cells can undergo both self-renewal (proliferation) and differentiation. Proliferation can be measured by the colorimetric MTT assay. Metabolically active cells can reduce MTT and generate purple formazan which can be colorimetrically measured. Accurate quantification of the proliferation rate is assumed due to the linear relationship between the cell number and the signal produced. Incubating with forskolin and IBMX greatly decrease the rate of cell proliferation, and increasing the concentration does not significantly change the proliferation rate (Fig 5). This result indicates that elevated cAMP level slowed the rate of stem cell proliferation, which may be due in part to the switch to differentiation.

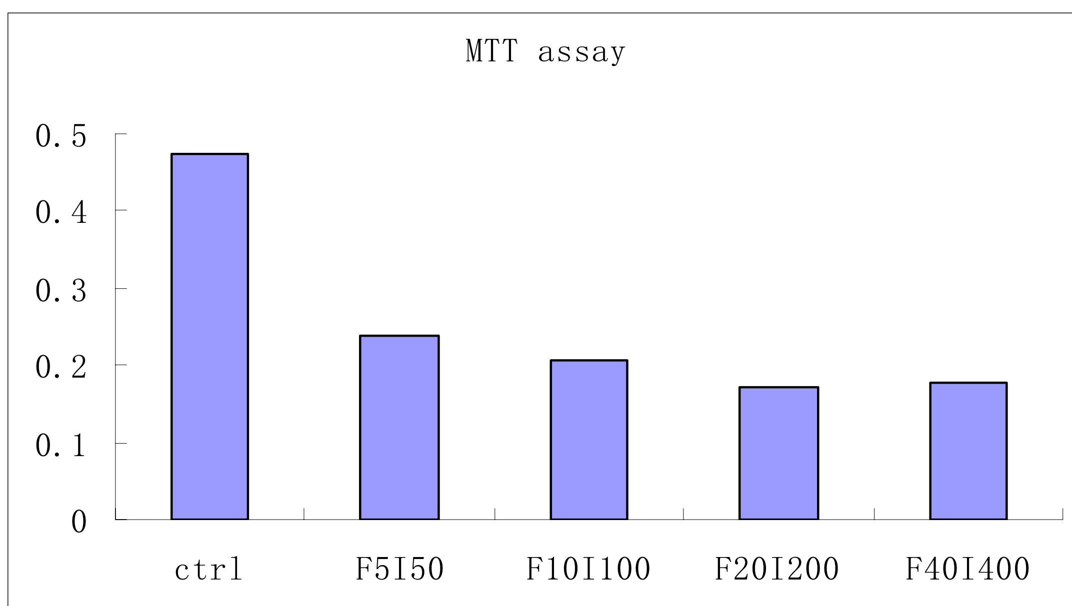


Fig 5 Cell proliferation rate of rMSCs incubated with or without forskolin and IBMX, at different concentrations. Forskolin and IBMX decreased the rate of cell proliferation, in a concentration independent manner.

Future work

Stem cells have the ability to maintain a balance between proliferation and differentiation. Environmental changes that induce intracellular signals will lead to the re-programming of gene expression and cause a switch towards differentiation. Stem cell exits cell cycle and prepares itself for differentiation. The two events, proliferation and differentiation, are closely related. As one of the crucial cell cycle regulators, cyclin D1 participates in G1/S phase transition by association with CDK4/6 (cyclin dependent kinase 4/6) and promote cell cycle progression. Regulation of cyclin D1 by CREB, ICER and NF-κB may partly account for slowed proliferation and how stem cells are promoted toward differentiation upon treatment with cAMP increasing agents. Work is underway to study the transcriptional regulation of

cyclin D1 by CREB, ICER and NF-κB in the cAMP induced neural differentiation of rMSCs. We hypothesize that cAMP induce differentiation of rMSCs by (1) direct inhibition of cyclin D1 transcription by ICER (2) Indirect inhibition of cyclin D1 transcription by down-regulation of NF-κB activity, which lowered proliferation rate by delaying G1/S phase transition and promotes stem cells toward differentiation. In addition, mathematical modeling of how transcription of cyclin D1 is regulated will be modeled by a set of differential equations according to the following figure (Fig 6). Sensitivity analysis will be performed to determine the sensitive parameters that would affect the output (cyclin D1 mRNA level) significantly. These parameters will be manipulated to guide the cell fate determination: proliferation versus differentiation.

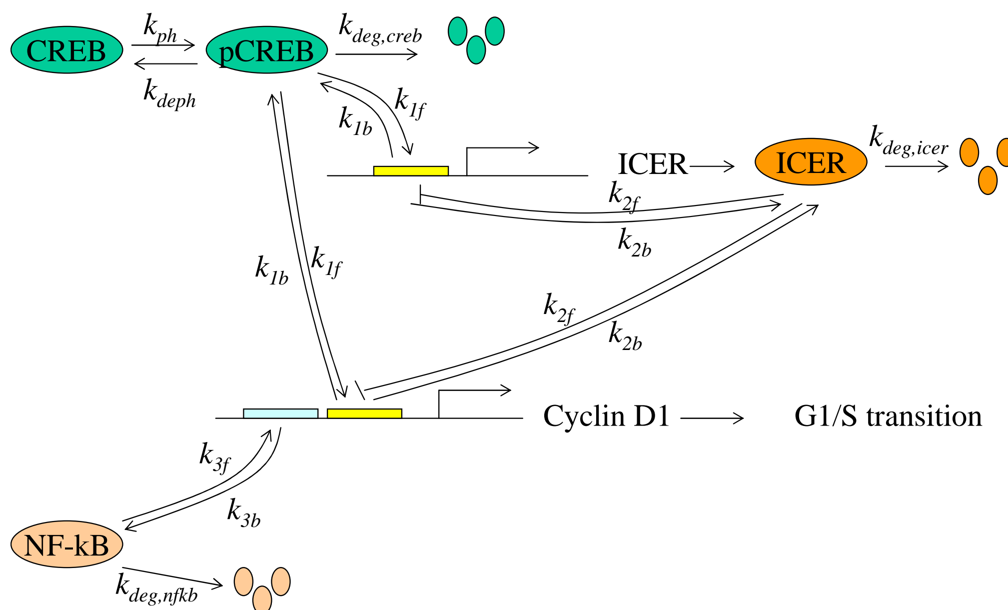


Fig 6 Proposed model of regulation of cyclin D1 expression by CREB, ICER and NF-kB.

Conclusion

The ability of MSCs to differentiate into neural cells gives the possibility that patients can use their own adult stem cell to cure neural degenerative diseases such as Parkinson disease. While it is known that cAMP can induce neural differentiation of MSCs, the transcriptional regulation of how stem cells differentiate is still poorly understood. Since proliferation and differentiation are two closely related events for stem cells, we are approaching investigation from the cell cycle regulation of stem cells to identify possible mechanisms that govern stem cell fate. Cyclin D1 is a good candidate due to its important role in regulating G1/S phase transition as well as its transcriptional regulation by CREB family proteins, which are involved in cAMP signaling. Since cyclin D1 also contains a κB motif and has been shown to be positively regulated by NF-κB, the role of NF-κB in modulating stem cell fate is also considered. Thus far, we have determined that elevated cAMP lowered proliferation. How cyclin D1 comes is involved and promotes differentiation is currently underway.

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