http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 12 (4): 324-331, 2009
ISSN 1028-8880

Kinetics of Gene Expression During Exposure of Mouse Stem Cells to Activin A

[1]Mahmoud Hashemi-Tabar, [1]Mahmoud Orazizadeh, [1]Ali Ghanbari and [2]Fereshteh Negad Dehbashi
[1]Cell and Molecular Research Centre, School of Medicine,
Jondishapour University of Medical Sciences, Ahwaz, Iran
[2]Department of Anatomy, School of Medicine, Kermanshah University of Medical Sciences,
Kermanshah, Iran

Abstract: This study aimed to evaluate the pattern of gene expression induced by activin A in mouse Embryonic Stem Cells (ESCs). Mouse ES cells cultured in undifferentiated state by leukemia inhibitory factor and feeder layer cells. Following removing these two anti differentiation factors for 5 days and forming Embryoid Bodies (EBs), the cells divided to 8 equal cells per groups. Differentiation procedure was performed in a two staged protocol; Formed EBs for 4 days (Stage one); expanded differentiated ESCs on gelatin coated dishes for one week (stage two). In the stage one, the media of groups 2-7 contained 10, 30 and 100 ng mL^{-1} Activin A. The media in stage two was the same for all groups and contained only Fetal Bovine Serum (FBS). The expression of undifferentiated, ectoderm, mesoderm and endoderm markers were compared with relative RT-PCR method and statistically analyzed. The expression of an undifferentiating marker; Nanog was increased in the Activin A treated groups of stage one. The expression of OCT4 reduced in Activin A treated groups in stage two. In the stage one, the expression of Nodal increased by Activin A. expression of sonic hedgehog (Shh) was suppressed in Activin A treated groups of both stages. In stage two, there were significant decrease for the expression of mesoderm (Brachyury) and Nodal and visceral endoderm (GATA4) markers ($p<0.01$). The expression of definitive endoderm markers (PDX1, TAT) showed significantly increased in Activin A treated groups ($p<0.01$). Activin A induced differentiation in high concentration by imbalance in undifferentiating markers. Nodal has a dual role, undifferentiating effect and regulation of visceral endoderm towards definitive endoderm. Overexpression of Nanog, alteration in the expression of Nodal and Shh inhibition are three mechanisms for explanation of differentiation induced by activin A in ES cells. These mechanisms induces cascade of gene expression that commits ESCs towards definitive endodermal cells.

Key words: Activin A, definitive endoderm, nanog, sonic hedgehog, stem cells

INTRODUCTION

Activin A belongs to TGF-β superfamily which controls many cellular processes such as proliferation, death and differentiation (Massagué *et al*., 2008).

The role for Activins in mesoderm formation and other stages of early mouse development has been recently investigated. Moreover, Activin signals establish the embryonic axes by mediating dorsoanterior mesoderm differentiation (Albano *et al*., 1993; Kessler and Melton, 1994; Smith *et al*., 1990).

Embryonic stem cells (ESCs) are able to contribute to all somatic tissues of the developing animal. This potential of ES cells represents them as an *in vitro* model for studding the development of mammals (Kao *et al*., 2008; Yamada *et al*., 2008).

Several studies showed the effects of activin A on differentiated states of ES cells but there are many controversies in this field. Indeed, some studies represent Activin A as an agent for maintaining ES cells in undifferentiated state (James *et al*., 2005; Vallier *et al*., 2005) while some studies claimed mesodermal differentiation of ES cells following treatment with Activin A (Albano *et al*., 1993; Johansson and Wiles, 1995).

On the other hand, for endodermal differentiation of ES cells many protocols are designed based on application of Activin A. These protocols deal with application of serum (Ibii *et al*., 2007) or serum free condition during treatment of ES cells for endoderm derived tissues such as pancreas (Nakanishi *et al*., 2007; Shi *et al*., 2005; Shim *et al*., 2007) or PDX1 expressing progenitors of pancreas (Shiraki *et al*., 2008).

Corresponding Author: Ali Ghanbari, Cell and Molecular Research Center, School of Medicine,
Jondishapour University of Medical Sciences, Ahwaz, Iran
Tel: 98-(0)611-336-7543 Fax: 98-(0)611-333-2036

The expression manner of markers is important landmark that is monitored for evaluating the role of Activin A in these experiments. For this reason, the expression of undifferentiated, ectodermal, mesodermal and endodermal markers are evaluated during cultivation of ES cells.

Present study aimed to investigate the gene expression of ES cells during exposure to activin A in the presence or absences of serum.

MATERIALS AND METHODS

In 2008, the study was conducted at the Cell and Molecular Research Centre, Faculty of Medicine, Department of Anatomy, Jondishapour University of Medical Sciences, Ahwaz, Iran.

***In vitro* differentiation of ES cells:** Mouse ES R_1 was maintained undifferentiated on mitomycin C (10 μg mL^{-1}) inactivated (Sigma # M0503) mouse embryonic fibroblast cells (MEFs) as feeder layer. The media was DMEM/F12 (Gibco# 21331-020) supplemented with 10% ESC-qualified fetal bovine serum (FBS) (Gibco# 10439-024), 1,500 units mL^{-1} leukemia inhibitory factor (LIF) (Sigma# L5283), 1% nonessential amino acid (Gibco# 11140-035)s, 2 mmol L^{-1} L-glutamine (Gibco# 25030-024), 0.1 mM/l β-mercaptoethanol (Sigma# M7522) and 100 units penicillin/100 μg streptomycin/mL (Gibco# 15070-063) (Fig. 1a).

MEFs were prepared from embryos at days 13.5-14.5 after gestation. They were decapitated and all inner organs, spinal cord and extremities were removed. The remaining tissue was cut into small pieces and incubated in 1 mL trypsin 0.5%/ EDTA (Invitrogen# 25300-054) for 30 min at 37°C. Then the trypsin was inactivated with 10 mL of DMEM/F12 containing 10% fetal bovine serum and following centrifuging and removing the media, the pellet was resuspended in fresh feeder media and plated on 25 cm^2 culture flasks (BD Falcon™# 355000). To initiate differentiation, ES cells were removed from feeder layer with 0.05% trypsin and 0.5 mM EDTA (Gibco # 25300-054) and counted in a Neubauer chamber to divide to 8 groups by equal numbers (2×10^6 cells/60-m dish). The clusters of ES cells were transferred into low cell-binding dishes. They were cultured at floating condition in the media which was the same as undifferentiated stem cells but without LIF to begin to form embryoid bodies (EBs). The ESCs cultured in this condition for five days and the media renewed every two days.

Afterwards, the formed EBs were cultured at floating condition for further 4 days in different media as fallows with daily media exchange (Stage one) (Fig. 1b):

- **Group 1:** DMEM/F12+10%FBS
- **Groups 2, 3 and 4:** DMEM/F12+10% FBS+ activin A (338-AC R and D system); 10 ng mL^{-1}, 30 ng mL^{-1}, 100 ng mL^{-1} respectively by the groups,
- **Groups 5, 6 and 7:** Knockout stem cell media (Gibco# 10829) +10% Knockout serum replacement (KOSR) (Gibco# 10829) + Activin A; 10 ng mL^{-1}, 30 ng mL^{-1}, 100 ng mL^{-1}
- **Group 8:** Knockout stem cell media +10% knockout serum replacement (KOSR)

After 4 days of suspension culture, total RNA of half of EBs of all groups was extracted for RT-PCR analysis. Another half of the EBs of all groups was allowed to differentiate for 1 week further in gelatin coated surfaces (0.1%) in DMEM/F12 containing 10% FBS with daily media changes (Stage two) (Fig. 1c). Total RNA of the differentiated ES cells of the stage two was extracted for RT- PCR analysis. During all periods of the protocol, ESCs were incubated at 37°C and 5% CO_2. For more illustration, the protocol is shown in Fig. 2.

RNA extraction, cDNA synthesis, and PCR: Total RNA was prepared using the RNA-easy kit (Qiagen, Courtaboeuf Cedex, France # 74134) and used as a template for reverse transcription-polymerase chain reaction (RT-PCR) which was performed using One- Step RT-PCR kite (Qiagen # 210212) according manuscript

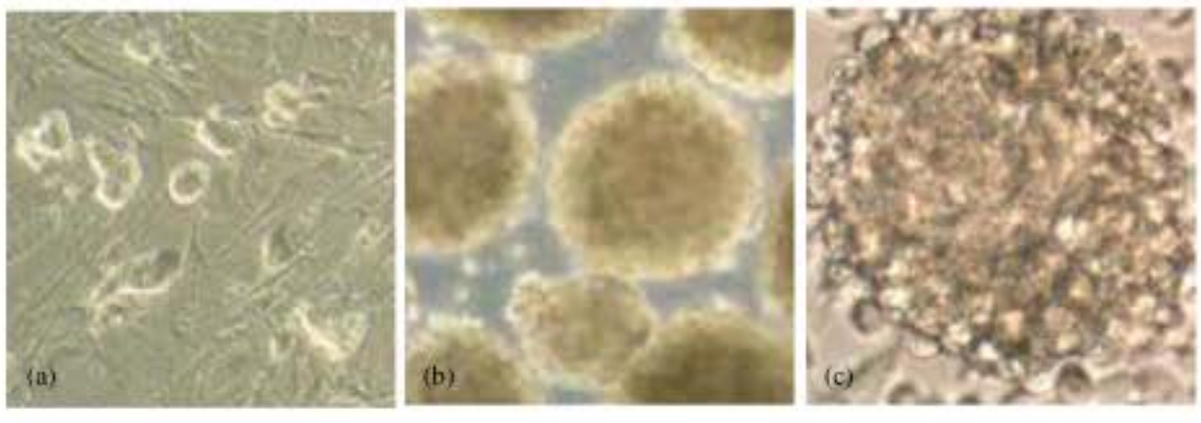

Fig. 1: Micrographs of mouse R_1 stem cells in phase contrast microscope. (a) Undifferentiated stem cell colonies on MEFs (10 X), (b) Differentiated embryo- like (Embryoid bodies) in suspension state (40 X) and (c) Expanded EBs on gelatin coated surfaces (100 X)

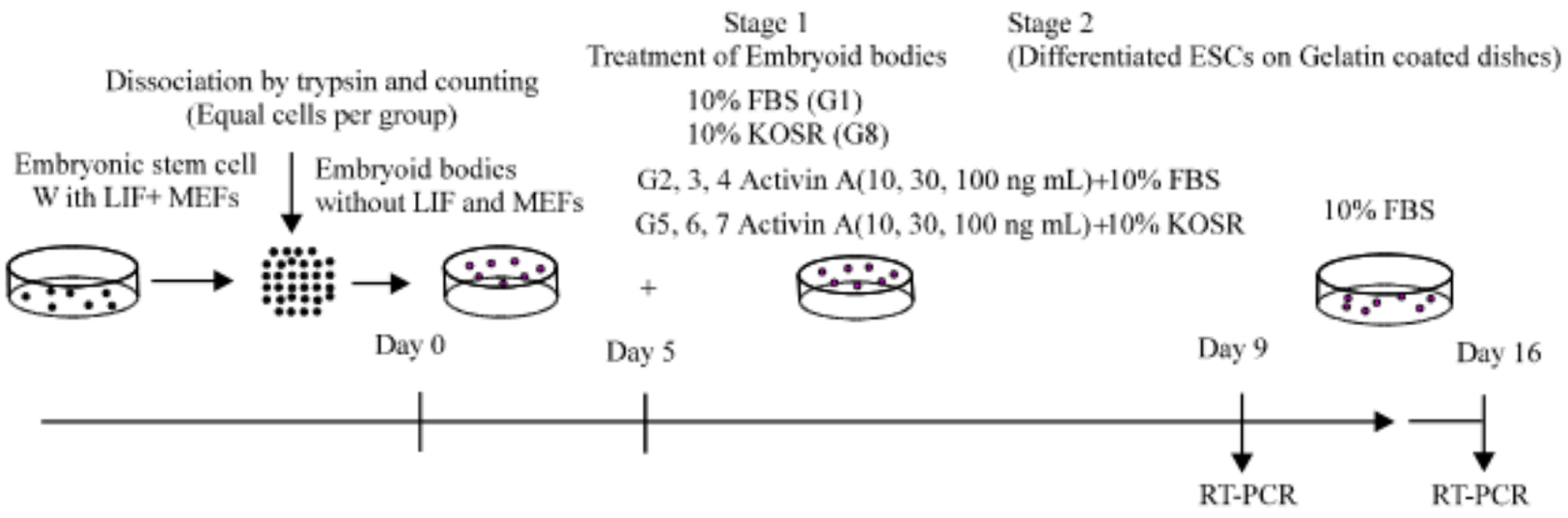

Fig. 2: The stages and different media for the groups of this study. Embryonic stem cells maintain undifferentiated in the presence of LIF and MEFs. Following removing these two antidifferentiating factors, the ESCs undergo differentiation after dissociation with trypsin. The ESCs cultured in floating condition for inducing embryoid bodies. After 5 days which EBs was induced, experimental groups (G 2-7) treated with some concentration of Activin A for four days (Stage one). Then, differentiated ESCs transferred to gelatin coated dishes for 1 week with the same media for all groups (Stage two). Total RNA was extracted at the end of stage one; Embryoid bodies and stage two; Expanded EBs for RT-PCR analysis. The study prolonged for 16 days

Table 1: Marker expression in this study

Gene	Forward sequence	Backward sequence	Annealing time	Size (bp)
GAPDH	ACCTCAACTACATGGTCTAC	TTGTCATTGAGAGCAATGCC	58	1801
Oct4	GGCGTTCTCTTTGGAAAGGTGTTC	CTCGAACCACATCCTTCTCT	58	293
Nanog	AGGGTCTGCTACTGAGATGCTCTG	CAACCACTGGTTTTTCTGCCACCG	60	278
GATA4	CTCCTACTCCAGCCCCTACC	GTGGCATTGCTGGAGTTACC	58	591
TAT	CAGAGGACTTGGTGGAGGAG	CACGGCTAGACACAGCTCAA	58	551
PDX	CTTAGCGTGTCGCCACAGCCCTCCA	CAGCCGCCTTTCGTTATTCT	58	472
Brachyury	CATGTACTCTTTCTTGCTGG	GGTCTCGGGAAAGCAGTGGC	60	313
Nestin	GGACAGGACCAAGAGGAACA	TCCCACCTCTGTTGACTTCC	58	599
Shh	GGCAGATATGAAGGGAAGAT	ACTGCTCGACCCTCATAGTG	59	260
Nodal	GACAGAAGCCAACTGTG	AGTGGCTTGG	60	223

instructions. To achieve the optimized cycle number which all the markers have shown log phase before plateau state, we have checked the expression of all markers between 20-40 cycles.

Then we selected 30 cycles, according to pilot study and to Stern-Straeter *et al*. (2008) for more confidence. Primer pairs, amplicon sizes, and annealing time are shown in Table 1.

Relative reverse transcription–polymerase chain reaction analysis: The expression of these target genes was quantified against that of the internal reference gene (GAPDH). Semi-quantitative reverse transcription-polymerase was carried out based on the comparison of CT at constant fluorescence intensity. Each PCR was performed under linear conditions with GAPDH used as an internal standard. Products were electrophoresed on a 1.5% agarose gel. The gels were stained with ethidium bromide (10 μg mL^{-1}) and photographed on an ultraviolet transilluminator (UVIdoc; Uvitec, Cambridge, UK). The gel images were analyzed using the UVIb and- map program (Uvitec). Semi-quantitative RT-PCR values were presented as a ratio of the specified gene signal divided by the GAPDH signal. The RT-PCR was performed as three individual replicates (Al-Bader and Al-Sarraf, 2005; Stern-Straeter *et al*., 2008).

Statistical analysis: The data of RT-PCR ratio values was analyzed using general linear model (GLM) frequency and correlation procedures in SPSS software (SPSS, Cicago, IL, USA). Results are expressed as the Mean±SD.

RESULTS

In this study, we considered the groups 1 and eight as control groups that in two stages of cultivation received only FBS for group one and KOSR in stage two for group eight. The others groups (2-7) which received Activin A in stage one are considered as experimental groups.

To determine the existence of undifferentiated ESCs, we monitored the expression of two pluripotency markers; OCT4 and Nanog in two stages of cultivation of ESCs as showed in Fig. 3. The profiles of the relative expression levels of OCT4 and Nanog showed (Fig. 4a, b).

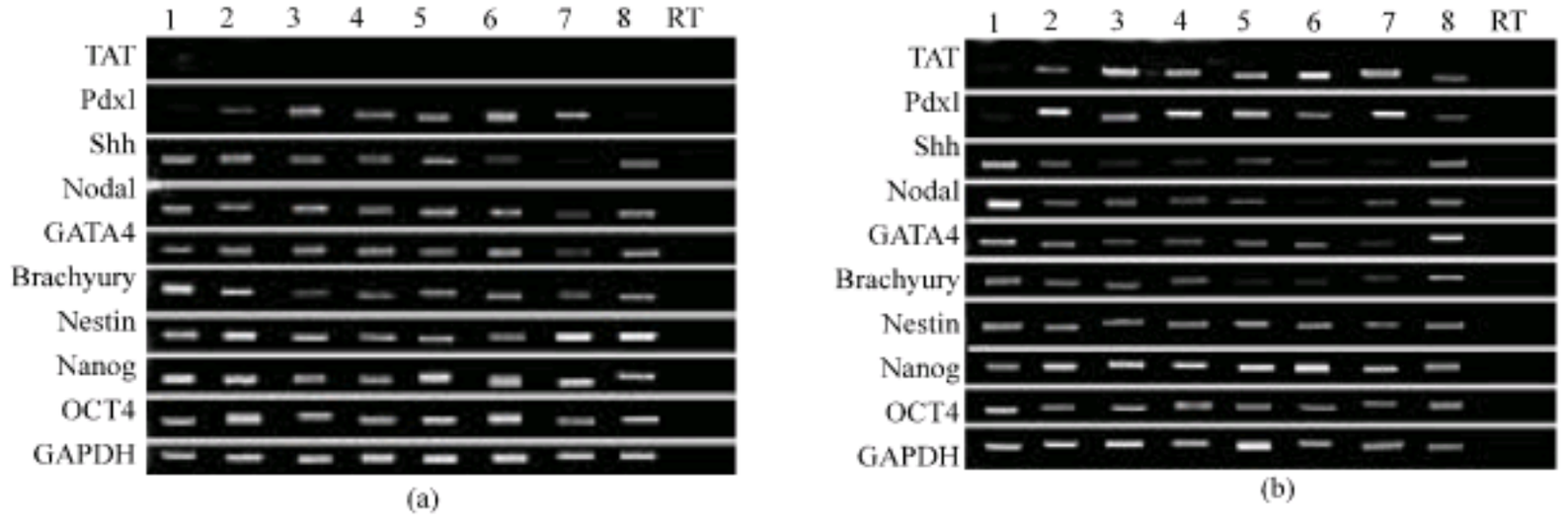

Fig. 3: Temporal expression pattern of genes by Reverse transcription polymerase chain reaction (RT-PCR) in eight experimental groups. (a): Stage one, (b): Stage two

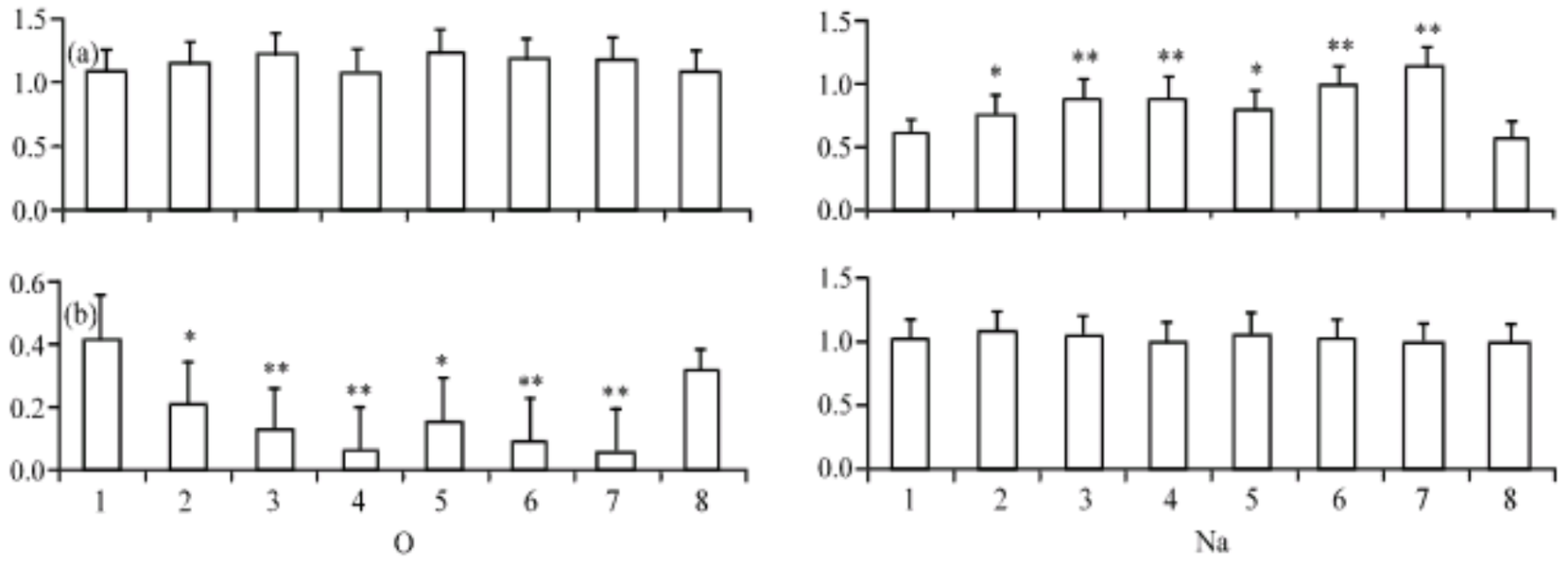

Fig. 4: Profiles of the expression of undifferentiating markers in eight groups of this study divided in two parts; (a) Stage one; Formation of embryoid bodies and (b) stage two; Expansion of differentiated ESCs; (O) OCT4 and (Na) Nanog (*p<0.05; **p<0.01)

The expression manner of OCT4 in the stage one was the same for all groups including FBS+Activin A and KSOR+Activin A and non Activin A containing groups. On the other hand, it was surprisingly observed higher expression of Nanog in Activin A treated groups. It was significant difference in groups 2 and 5 with $p<0.05$ and in groups 3, 4, 6 & 7 with $p<0.01$. Moreover, the most prominent expression of Nanog was observed in KSOR+ Activin A 100 ng mL^{-1} (Group 7). This data indicated that Activin A did not reduce the expression of this two undifferentiating marker during EB formation (Fig. 4a). In contrast to stage two, there was a significant reduction in expression of OCT4 in the groups that treated with activin A. Moreover, the expression of OCT4 was reduced significantly in KOSR+Activin A groups; 6 and 7 However, the expression of OCT4 was observed at final stage for all groups (Fig. 4b). The expression of Nanog in stage two did not showed statistical differences in all groups (Fig. 4b).

To verify the induction of differentiation, the expression of ectoderm (Nestin), mesoderm (Brachyury) and endoderm (Nodal, GATA4, PDX1 and TAT) markers was evaluated in two stages of cultivation for all groups, respectively (Fig. 5).

In stage one, there were not statistically significant differences in the expression of differentiating markers but for Shh, Nodal and PDX1. The expression of Shh significantly reduced in groups 6, 7 ($p<0.01$) and 4 ($p<0.05$) while expression of Nodal increased for groups 3 ($p<0.05$), 4 ($p<0.01$), 6($p<0.05$) and 7 ($p<0.01$) and expression of PDX1 increased in all Activin A involved groups ($p<0.01$) (Fig. 5a). This data showed the effect of Activin A on suppression of Shh and promotion of Nodal and PDX1 in a dose dependent manner during EB formation. In stage two (Fig. 5b), the expression of ectoderm marker (Nestin) did not show significant differences while the expressions of pancreatic progenitors (PDX1) and liver progenitors (TAT) markers which both belong to endoderm lineage were elevated in Activin A treated groups with a dose dependent manner ($p<0.01$).

The expression of Nodal and Visceral endoderm (GATA) and mesoderm marker (Brachyury) also were reduced in a dose dependent manner in all Activin A involved groups ($p<0.01$).

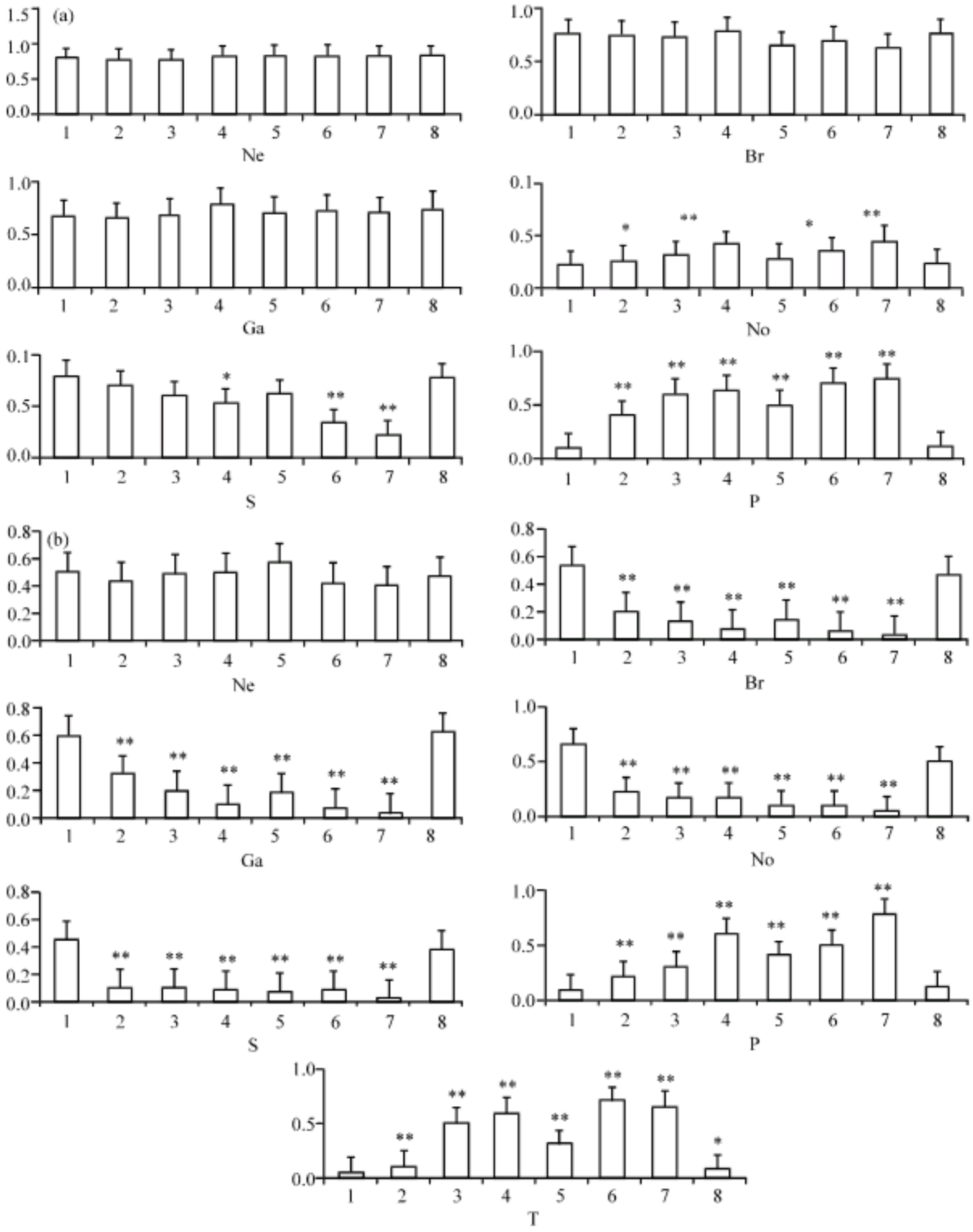

Fig. 5: Profiles of the expression of differentiating markers in eight groups of this study divided in two parts; (a) Stage one; Formation of embryoid bodies and (b) stage two; Expansion of differentiated ESCs. Ne: Nestin, s: Shh, P: PDX1, Br: Brachyury, Ga: GATA4 and No: Nodal (*p<0.05; **p<0.01)

Moreover, in the absence of serum (FBS); groups 5-7, Activin A more reduces the expression of differentiating markers in comparison with Activin A+FBS groups; 2-4. Thus, in the protocols of differentiation derived by Activin A, it is better to use KOSR besides FBS. The most differentiation effect of Activin A was observed in group 7 which ESCs treated with Activin A (100 ng mL^{-1}) + KOSR.

DISCUSSION

The data showed the expression of OCT4 was reduced by Activin A; whether there was no interruption in OCT4 positive cells. This issue confirms other studies that show Activin A does not put an end to the expression of OCT4 (Fujikawa *et al*., 2005). Therefore we observed after prolonged cultivation of EBs, the

expression of OCT4 did not reduced and cell transplanted therapy by differentiated ESCs causes teratoma in donor animals (Berrill *et al*., 2004). Thus, it is consistency wit our findings that the existence of undifferentiated cells expressing OCT4 is a main problem for transplantation of differentiated cells derived from ES cells.

The data showed Activin A increased the expression of Nanog with a dose dependent manner. It is partly due to TGFb/Activin signaling which leads to mESCs in undifferentiated state (Beattie *et al*., 2005). The TGF β /Activin signaling is transmitted by the phosphorylation of SMAD2/3, which is abundant in undifferentiated human ESCs (Xiao *et al*., 2006). Nanog is a candidate gene as a target for Activin signaling. In this issue, It is showed that SMAD2/3 bind directly to the NANOG proximal promoter in human ESCs (Xu *et al*., 2008).

On the other hand, the balance of OCT4 and Nanog, which constitute the core intrinsic factors are required to sustain the selfrenewing and pluripotent cellular state and when it disrupts the ES cells to be differentiated (Niwa, 2007). Therefore, the imbalance of OCT4-Nanog that was observed in stage one of this study may explain the increased level of differentiating markers.

In the case of undifferentiating property, some studies confirm that transforming growth factor β (TGFb)/Activin promote maintaining ES cells in undifferentiated states with increasing the expression of nodal (James *et al*., 2005; Vallier *et al*., 2005). This issue can explain the overexpression of Nodal at the end of EB formation but at the end of stage two the expression of nodal was reduced by Activin A.

Temporal gene expression of human embryonic cells shows the overexpression of Nodal during formation of the EBs but reduces after 10 days of cultivation of EBs (Dvash *et al*., 2004). Thus, in the presence of Activin A, the up regulation of Nodal is an mechanism for maintaining ES cells in undifferentiated state while following removing this extra Nodal expression there is more reduction of this marker at the end of the protocol.

In this study, the expression of Gata4 which is a marker of visceral endoderm was reduced by Activin A while PDX1 (marker of progenitors of pancreas) and TAT (marker of progenitor of liver) was increased.

In vivo and *in vitro* experiments clear that Nodal expression transiently occurs in visceral endoderm of mouse embryos before or after formation of primitive streak (Varlet *et al*., 1997).

Thus in agreement with the others, Activin A increases the expression of Nodal in first stage of cultivation of ES cells while reduces its expression for inducing definitive endoderm besides visceral endoderm. We suggest that some of Nodal over expression observed in stage one may due to the expression of this marker in visceral endoderm of the EBs that induced by Activin A. Moreover, fate of the ESCs shifts towards definitive endoderm by the regulation derived from this extra Nodal expression in stage one. This idea derived from in vivo studies that show a prominent role for patterning of endoderm by Nodal (Bielinska *et al*., 1999).Thus, monitoring the position of Nodal marker in Stage one (EB formation) in comparison with stage two (Expansion of differentiated ESCs) can be an attractive field for research.

On the other hand, reduction of Shh which was induced by Activin A in this study is needed for inducing PDX1 expression in protocols for generation of insulin producing cells from ESs that explain the strong expression of PDX1 in this study (Mfopou and Bouwens, 2008).

Similarly, addition of hedgehog pathway inhibitors (anti-sonic hedgehog antibody or cyclopamine) to culture medium led to increased expression of both endocrine and exocrine pancreas markers (Leon-Quinto *et al*., 2004; Skoudy *et al*., 2004). It is noticeable that expression of TAT which is a marker for progenitor of liver was also increased in Activin A treated groups in agreement with some protocols for induction of liver by Activin A (Hay *et al*., 2008a, b).

Recently, it showed that a transient tissue named as mesendoderm forms during differentiation of ES cells. Mesendoderm cells are in decision state for going to differentiate to mesoderm or endoderm (Tada *et al*., 2005). We suggest that, Shh inhibition, commits mesendoderm cells and besides ongoing mesoderm pathway they become endoderm marker expressing cells. This issue maybe an explanation for the suppression of Brachyury (Fig. 4) derived by Activin A. The recognition of the role of mesendoderm in the process of differentiation of stem cells provides new perspectives about the pattern of gene expression derived by Activin A.

The result show absence of serum is important and more Nanog expression occurs which leads to more expression in differentiating markers (Fig. 3, 4). Importantly, LIF is effectively only in media containing serum and the ES cells stay more in undifferentiated state in the presence of the serum. Thus, serum may contain some agents which maintain the ESCs in undifferentiated state (Ying *et al*., 2003). We suggest for eliciting serum during treatments of ES cells with Activin A for performing endoderm differentiation protocols.

Our data showed that Activin A does not change the expression of Nestin. This idea is in concern with some studies that examined no Nestin selection for producing pancreatic progenitors (Blyszuk *et al*., 2004).

Summing up, our data show that high concentration of activin A disrupts the expression balance of pluripotency markers. This issue may explain cascade of gene regulation. Indeed, the process followed by

overexpression of Nodal and suppression of Shh that leads to the formation of definitive endoderm besides visceral endoderm. These two mechanisms may explain the over expression of endoderm markers and restriction of mesoderm marker but it needs for treatment of ES cells with an instant Shh inhibitor agent to clear which of these two mechanisms leads ES cells to differentiate towards endoderm lineages. Mesendoderm cells may be a key target for each or both of these two supposed mechanisms.

ACKNOWLEDGMENTS

Authors acknowledge the cell and molecular research centre for their corporations staffs. Dr. Massoud Soleimani (Tarbiat Modarres University, Tehran, Iran) for gifting the R_1 cell line. This work was supported by a research grant (no U-86071) from Jondishapour University of Medical Sciences.

REFERENCES

Albano, R.M., N. Groome and J.C. Smith, 1993. Activins are expressed in preimplantation mouse embryos and in ES and EC cells and are regulated on their differentiation. Development, 117: 711-723.

Beattie, G.M., A.D. Lopez, N. Bucay, A. Hinton, M.T. Firpo, C.C. King and A. Hayek, 2005. Activin a maintains pluripotency of human embryonic stem cells in the absence of feeder layers. Stem. Cells, 23: 489-495.

Berrill, A., H.L. Tan, S.C. Wuang, W.J. Fong, Andre, B.H. Choo and K.W.O. Steve, 2004. Assessment of stem cell markers during long-term culture of mouse embryonic stem cells. Cytotechnology, 44: 77-91.

Bielinska, M., N. Narita and D.B. Wilson, 1999. Distinct roles for visceral endoderm during embryonic mouse development. Int. J. Dev. Biol., 43: 183-205.

Blyszuk, P., C. Asbrand, A. Rozzo, G. Kania, L. ST-Onge, M. Rupnik and A.M. Wobus, 2004. Embryonic stem cells differentiate into insulin-producing cells without selection of nestin-expressing cells. Int. J. Dev. Biol., 48: 1095-1104.

Dvash, T., Y. Mayshar, H. Darr, M. McElhaney and D. Barker *et al*., 2004. Temporal gene expression during differentiation of human embryonic stem cells and embryoid bodies. Hum. Reprod., 19: 2875-2883.

Fujikawa, T., S.H. Oh, L. Pi, H.M. Hatch, T. Shupe and B.E. Petersen, 2005. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am. J. Pathol., 166: 1781-1791.

Hay, D.C., J. Fletcher, C. Payne, J.D. Terrace and R.C. Gallagher*et al*., 2008a. Highly efficient differentiation of hESCs to functional hepatic endoderm requires Activin A and Wnt3a signaling. Proc. Natl. Acad. Sci. USA., 105: 12301-12306.

Hay, D.C., D. Zhao, J. Fletcher, Z.A.D. Hewitt and A. McLean *et al*., 2008b. Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development *in vivo*. Stem. Cells, 26: 894-902.

Ibii, T., H. Shimada, S. Miura, E. Fukuma, H. Sato and H. Iwata, 2007. Possibility of insulin-producing cells derived from mouse embryonic stem cells for diabetes treatment. J. Biosci. Bioeng., 103: 140-146.

James, D., A.J. Levine, D. Besser and A. Hemmati-Brivanlou, 2005. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development, 132: 1273-1282.

Johansson, B.M. and M.L. Wiles, 1995. Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. Mol. Cell. Biol., 15: 141-151.

Kao, C.F., C.Y. Chuang, C.H. Chen and H.C. Kuo, 2008. Human pluripotent stem cells: current status and future perspectives. Chin. J. Physiol., 51: 214-225.

Kessler, D.S. and D.A. Melton, 1994. Vertebrate embryonic induction: Mesodermal and neural patterning. Science, 266: 596-604.

Leon-Quinto, T., J. Jones, A. Skoudy, M. Burcin and B. Soria, 2004. *In vitro* directed differentiation of mouse embryonic stem cells into insulin-producing cells. Diabetologia, 47: 1442-1451.

Massagué, J.A., 2008. Very private TGF-beta receptor embrace. Mol. Cell, 29: 149-150.

Mfopou, J.K. and L. Bouwens, 2008. Hedgehog signals in pancreatic differentiation from embryonic stem cells: Revisiting the neglected. Differentiation, 76: 107-117.

Nakanishi, M., T.S. Hamazaki, S. Komazaki, H. Okochi and M. Asashima, 2007. Pancreatic tissue formation from murine embryonic stem cells *in vitro*. Differentiation, 75: 1-11.

Niwa, H., 2007. How is pluripotency determined and maintained? Development, 134: 635-646.

Shi, Y., L. Hou, F. Tang, W. Jiang, P. Wang, M. Ding and H. Deng, 2005. Inducing embryonic stem cells to differentiate into pancreatic ß cells by a novel three-stage approach with activin A and all-trans retinoic acid. Stem. Cells, 23: 656-662.

Shim, J.H., S.E. Kim, D.H. Woo, S.K. Kim, C.H. Oh, R. McKay and J.H. Kim, 2007. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia, 50: 1228-1238.

Shiraki, N., T. Yoshida, K. Araki, A. Umezawa and Y. Higuchi*et al*., 2008. Guided differentiation of embryonic stem cells into Pdx1-expressing regional-specific definitive endoderm. Stem Cells, 26: 874-885.

Skoudy, A., M. Rovira, P. Savatier, F. Martin, T. Leon-Quinto, B. Soria and F.X. Real, 2004. Transforming growth factor (TGF)beta, fibroblast growth factor Biochem. J., 379: 749-756.

Smith, J.C., B. Price, M. Van and D. Huylebroeck, 1990. Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin A. Nature (London), 345: 729-731.

Stern-Straeter, J., G. Bran, F. Riedel, A. Sauter, K. Hörmann and U.R. Goessler, 2008. Characterization of human myoblast cultures for tissue engineering. Int. J. Mol. Med., 21: 49-56.

Tada, S., T. Era, C.C. Furusawa, H. Sakurai and S. Nishikawa *et al*., 2005. Characterization of mesendoderm: A diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development, 132: 4363-4374.

Vallier, L., M. Alexander and R.A. Pedersen, 2005. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci., 118: 4495-4509.

Varlet, I., J. Collignon and E.J. Robertson, 1997. Nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. Development, 124: 1033-1044.

Xiao, L., X. Yuan and S.J. Sharkis, 2006. Activin a maintains self-renewal and regulates fibroblast growth factor, wnt and bone morphogenetic protein pathways in human embryonic stem cells. Stem. Cells., 24: 1476-1486.

Xu, R.H., T.L. Sampsell-Barron, F. Gu, S. Root and R.M. Peck *et al*., 2008. NANOG is a direct target of TGFb/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell, 3: 196-206.

Yamada, S., T.J. Nelson, R.J. Crespo-Diaz, C. Perez-Terzic and X.K. Liu *et al*., 2008. Embryonic stem cell therapy of heart failure in genetic cardiomyopathy. Stem Cells, 26: 2644-2653.

Ying, Q.L., J. Nichols, I. Chambers and A. Smith, 2003. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell, 115: 281-292.