Cloning of Oct3/4 gene in embryonic stem cells

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ABSTRACT

Embryonic stem cells (ESCs) are pluripotent, self-renewing cells. These cells can be used in applications such as cell therapy, drug discovery, disease modelling, and the study of cellular differentiation. In this experimental study embryonic stem cells cultured in the laboratory and were amplified. Total RNA was extracted from cells and converted to cDNA. The replication factor Oct3/4 gene was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into the pTZ57R/T vector. Legated product had been transformed into susceptible bacteria and transformed bacteria were screened on a selective medium. Plasmids extracted from bacteria and enzyme digestion to confirm the sequencing was performed. Results of enzyme digestion were sequenced. Cloned gene can prepare a gene cassette to produce stem cells from somatic cell.

Keywords: Cloning; Embryonic Stem Cells; Oct3/4 Gene.

INTRODUCTION

Human ES cells may become an unlimited source of cells or tissues for transplantation therapies involving organs or tissues such as liver, nervous system, pancreas and blood. Despite a tremendous interest in ES cells, relatively little is known about what defines their pluripotency and what drives ES cells to differentiate into specific cell types. The development of the mammalian embryo is controlled by regulatory genes, some of which regulate the transcription of other genes [1 & 2]. These regulators activate or repress patterns of gene expression that mediate phenotypic changes during stem cell differentiation. Oct4 (also known as Oct-3) belongs to the POU (Pit- Oct-Unc) transcription factor family [2].The POU family of transcription factors can activate the expression of their target genes through binding an octameric sequence motif of an AGTCAAAT consensus sequence [1-3]. Recent evidence indicates that Oct4 is almost exclusively expressed in ES cells [4 & 5]. During embryonic development, Oct4 is expressed initially in all blastomeres. Subsequently, its expression becomes restricted to the ICM and down-regulated in the TE and the primitive endoderm. At maturity, Oct4 expression becomes confined exclusively to the developing germ cells [3- 6].

These cells can re-enter the developing embryo and contribute to all cell types of the embryo including germ line. Loss of pluripotency in ES cells is often accompanied by Oct4 down-regulation. Oct4 deficient mouse embryos fail to develop beyond the blastocyst stage due to the lack of pluripotent ICM [7], suggesting the critical role of Oct4 in maintaining ES cell pluripotency *in vivo*. However, Oct4 is not the only master gene that controls ES cell pluripotency. Oct3/4 is a transcription factor known to play a key role in the maintenance and self-renewal of pluripotent cells. Biochemically, Oct4 has been shown to be a DNA-binding protein with a bipartite POU/homeodomain encoded by a 324-amino acid open reading frame [8]. Oct4 relies on two trans-activation domains flanking the DNA-binding domain to exert its transcription activities [9].

Oct4 protein is synthesized in the cytosol and transported into the nuclei via a typical nuclear localization signal [10]. The nuclear localization signal of Oct4 is required for its transcription activity, and its ablation leads to the generation of a dominant-negative form of Oct4, which is capable of inducing ES cell differentiation by interfering with wild-type Oct4 activity [10]. ES cells cannot be established from Oct3/4 knockout embryos, while repression of Oct3/4 in ES cells results in differentiation into trophoblast-like cells, demonstrating the essential role played by

Oct3/4 in the maintenance of pluripotency.

Interestingly, Oct3/4 is also important in promoting differentiation, as its over-expression by as little as 50% results in differentiation of ES cells into mesoderm and endoderm. Together, these findings suggest that Oct3/4 expression levels require very tight regulation in ES cells. Oct3/4 has been shown to have a large number of target genes, many of which also possess regulatory elements for Sox2 and Nanog. Such genes are frequently up-regulated or down-regulated in ES cells, and encode proteins involved in ES cell signalling. Oct3/4 is known to co-operate with Sox2 to regulate a number of genes, including *Sox2* and *Oct3/4* in a positive feedback loop and *Nanog*. Binding sites for both Oct3/4 and Sox2 have also been found in a number of other genes specifically expressed in ES cells including *Fbx15* and *Lefty*. In addition, two regulatory elements exist, which act as stem-cell-specific enhancers of the *Oct3/4* gene. Many regulators are recruited to these elements; and shifts in the balance between positive and negative regulators may give rise to variation in the levels of *Oct3/4* expression, in response to external stimuli. Liver receptor homologue 1 (Lrh1) acts as a positive regulatory factor for *Oct3/4* while germ cell nuclear factor (Gcnf) acts as a repressor by recruiting Dnmt3 and promoting methylation of the *Oct3/4* promoter [9, 10].

MATERIALS & METHODS

Cell culture

In this study, human embryonic stem line, (hSCT1; Stem cell technology research centre, Tehran, Iran) was cultured in DMEM-F12 supplemented with 20% knockout serum replacement, 2 mM L-gluatamine, 1% nonessential amino acid, and 20 ng/ml human bFGF (all from Invitrogen), and 0.1 mM 2-mercaptoethanol (Sigma). Primary mouse embryonic fibroblasts (MEFs) were used as the feeder cells in passages 2-4. Monolayer of feeders were grown in Dulbecco modified Eagle medium (DMEM; GIBCO) supplemented with 15% fetal calf serum (FCS; GIBCO), 2 mM L-glutamine (GIBCO), 25 U/mL penicillin, and 25 µg/mL streptomycin (GIBCO), to confluence in a T-flask and treated with 10 µg/mL mitomycin-C (Sigma) for 2 h. After treatment, cells were washed extensively in PBS, detached with 0.25% trypsin–ethylene-diamine tetra-acetic acid (EDTA), and re-plated in gelatin-coated tissue culture dishes.

hESC experiments were performed on passage 12 from initial establishment. The cells were passaged every 3–5 days with 1 mg/ml collagen IV (Invitrogen). After PBS washing, the cells were dispersed by scraping and passaged on fresh inactivated MEFs with above mentioned culture medium.

Cloning of OCT3/4 gene

Total RNA of cells was extracted by RNA fast kit (provided by National Research Center of Genetic Engineering and Biotechnology, Tehran, Iran) based on using guanidine isothiosyanate. The efficiency of RNA extraction was confirmed by 1% agarose gel electrophoresis. Then, total RNA was stored in -70°C until use time. For accomplishment of reverse transcription (RT) reaction to obtain equivalent cDNA, we used expanded first strand cDNA synthesis kit (Roche Molecular Biochemicals, Germany). In this kit, the RNase activity of reverse transcriptase enzyme (MuLv, moloney murin leukemia virus) has been eliminated due to existence of a mutation in C-terminus of RT enzyme. The sequence of Oct3/4 gene related to Human embryonic stem cell was gained from Gen Bank followed by two pairs of primers were designed(Table 1).The primers were used to amplify and clone of the complete sequence of Oct3/4 gene.

Table1. The oligonucleotide primers used in the PCR assay

Primers	Sequence
Forward:	5́- CATATGGCGGGACACCTGGCTTCG -3́
Reverse:	5́- GCGGCCGCGTTTGAATGCATGGGAGAGCCC -3́

The annealing temperature for primers was set at 62°C. The extension time was 60 seconds. B actin gene in all reactions was used as internal control. Amplification was made in a total volume of 25 µl of reaction mixture containing 1µl of genomic DNA , 2.5 µl of 10× PCR buffer, 1.5 µl of $MgCl_2$ (50 mM), 200 µM dNTP(10mM), 1.5 µl of each primer (10 pmol) and 0.25 µl of Taq polymerase (5 unit/µl). By adding double-distilled water, the reaction mixture reached the Final volume 25 µl. A total of 32 cycles was performed with the first denaturation at 95 ℃ for 5 min, then 30 cycles at 95 ℃ for 30 seconds, 67 ℃ for 30 seconds and 72 ℃ for 30 seconds, and the final extension at 72 ℃ for 5 min. The amplified products were analyzed by electrophoresis with a 1% agarose gel followed by ethidium bromide staining and UV-transilluminator visualization.

After the end of reaction, the amount of 7 µl of the product was used to run on 1% agarose gel electrophoresis and then staining results were compared with marker 1kbp (Fermentase). After all proprietary products, PCR product purified and with the cloning vector pTZ57R / T were Lygated and transformed into *E. coli* DH5α. Ampicillin selection

and some control tests ensured the presence of the recombinant plasmid. PCR with specific primers was performed for some of the cloned hosts. The recombinant clonies in PCR reaction were sent for sequencing.

RESULTS

Result of Cell culture

The results of embryonic stem cells culture has been shown in Figure 1.

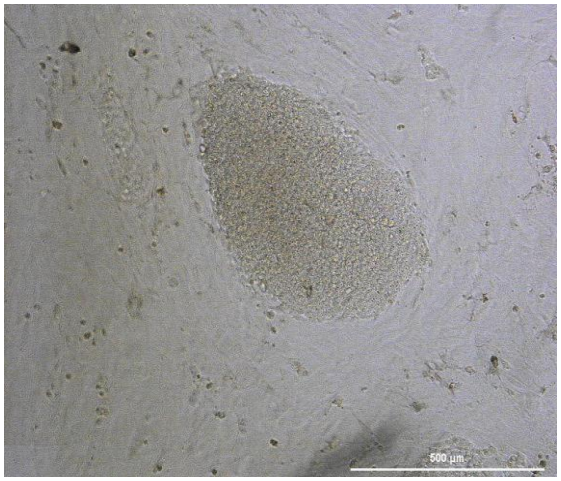

Figure 1. hSCT1 human embryonic stem cell line.

Results of Gene cloning

Proliferation area which code human Oct3/4 gene was provided in the presence of desired primers and 1070 bp PCR product of human Oct3/4 genes was obtained.

The 1070-bp PCR product was cloned in vector pTZ57R/T. PCR reaction of the clonies on selective culture indicates some of these recombinant clonies specific for hosts by using Oct3/4 primers. Lane M: DNA molecular weight marker, Lane 1: Recombinant clonies, Lane 2: Non recombinant clonies (Figure 2). Results of the sequencing on plasmids extracted from recombinant clonies and comparison with gene data bank confirmed cloning of these genes.

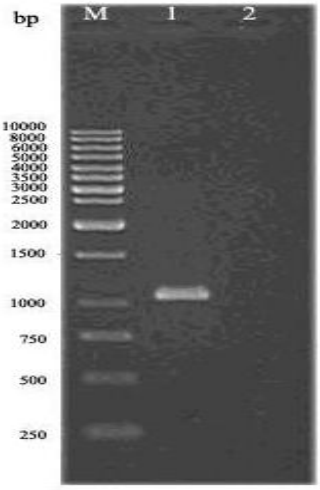

Figure 2. Polymerase chain reaction of the cloned

DISCUSSION

iPS cells, generated by introduction of Oct3/4, Sox2, Klf4 and c-Myc transcription factors into fibroblasts, share many properties with ES cells. Additionally, contribution of iPS cells to live adult chimeric animals and germline transmission clearly demonstrate that re-programming, to cells very similar to pluripotent ES cells, was achieved. Pluripotent stem cells (ESCs and iPSCs) are of great interest for basic research and clinical applications because they are uniquely capable of self-renewal and differentiation into a large number of cell types. These cells make it possible for the first time to analyze the epigenetic control of pluripotence and differentiation.

Yamanaka and co-workers surprised the scientific community when they reported that both mouse embryonic fibroblasts and tail tip fibroblasts could be reprogrammed to a pluripotent state similar to that observed in embryonic stem (ES) cells, by retroviral transduction of just four genes. The discovery of these 'induced pluripotent stem (iPS) cells' was generally regarded as a major development in stem cell research and gave new insights into the pathways involved in the maintenance of pluripotency. Due to the complexity of genetic and epigenetic changes involved in cell differentiation, it had been doubted if it would ever be possible to reprogramme somatic cells to pluripotency. With the first successful cloning experiments in mammals, it was verified that such reprogramming was indeed possible. However, the landmark discovery by Takahashi and Yamanaka less than a decade later signalled a development, which few expected so soon.

By definition, pluripotency is the ability of a cell to give rise to all cell types of an adult organism, without the self-organising capability to form the whole

organism. *In vivo*, pluripotency is observed in early embryos while *in vitro*, pluripotency may be maintained in ES cells. ES cells may be harvested from the inner cell mass (ICM) of blastocyst stage embryos. These cells, which were first isolated from mouse embryos, can proliferate indefinitely and possess the potential to develop in an unrestricted manner.

In culture, the pluripotency of mouse ES cells must be maintained by addition of factors such as leukaemia inhibitory factor (LIF), which promote proliferation while preventing differentiation. Human ES cells lines have also been generated, and their potential as donor sources of specialised cells in cell transplantation therapies has been widely acknowledged.

However, some major concerns remain for ES cell transplantation. Tissue rejection due to the patients, immune response represents a real limitation of the use of ES cells for transplantation. Another concern is that in the process of isolating ES cells, human embryos are inevitably destroyed. This has been a source of constant controversy since the development of the first human ES cell lines, and it has become an important ethical and political issue. These problems may, however, be overcome by reprogramming differentiated cells to an ES cell-like, pluripotent state. Such cells could be customised for individual patients and used in the treatment of disease.

Improvements in methodologies for detecting gene expression in the early embryo have led to the identification of several genes that may be involved in the regulation of early developmental events. These include genes encoding growth factors, their receptors, and numerous transcription factors, among which Oct4 seems to play a major role [11]. Oct4 (also known as OTF3, POU5F1) is a POU-domain, octamerbinding transcription factor expressed in both mouse and human embryonic stem cells (ESCs) and primordial germ cells (PGCs). Oct4 expression is necessary for the maintenance of pluripotentiality in ESC and PGC and it is down-regulated in all differentiated somatic cell types *in vitro* as well as *in vivo* [3].

Decreased Oct4 gene expression is accomplished via increased DNA methylation and structural changes involving the immediate upstream regulatory region[12]. The Oct4 gene is un-methylated in the blastula stage and subsequently undergoes *de novo* methylation at 6.5 days postcoitum, remaining modified in all adult tissues [11 & 12]. A similar pattern was found in human EC cell lines [2]. A similar relationship between expression and methylation pattern was recently found in *in vivo* samples of GCTs. In addition to its role in the maintenance of pluripotentiality, Oct4 may also play a role in maintenance of viability of the mammalian germline, functioning as a 'stem cell survival' factor [14 & 15]. Primordial germ cells lacking Oct4 expression have been shown to undergo apoptosis rather than differentiation [16].

Variations in Oct4 levels are responsible for most failures in somatic cell cloning [17 & 18]. These findings imply a possible different function of Oct4 in ESCs and PGCs, which may have an impact on the understanding of its role in the pathogenesis of GCTs. The fundamental role of Oct4 expression in the preservation of pluripotent cells in the inner cell mass of blastocysts has been demonstrated by specific disruption of the Oct4 gene in mice, showing that Oct4 deficiency in the mouse embryo results in failure of the inner cell mass to develop [11]. Moreover, a critical amount of Oct4 is needed for the self-renewal of normal ESCs, while both up- and down-regulation of Oct4 expression are responsible for divergent differentiation of these pluripotent cells [13].

Quantitative analyses in mouse ESCs show that high levels of Oct4 expression cause differentiation into primitive endoderm and mesoderm, whereas low levels determine loss of pluripotency and differentiation towards trophectoderm [20]. Recently, these results have been confirmed by analyses with Oct4-specific short interfering RNA, inducing knockdown of Oct4 in both mouse and human cell lines [14&15].Since Oct4 was first identified in EC cells and is expressed in all EC cell lines and because Oct4 confers pluripotentiality, as well as cell survival, to germ cells, the aberrant expression of this transcription factor might contribute to tumourigenesis in GCTs [3].

Indeed, Gidekel *et al* found that the level of Oct4 expression dictated the oncogenic potential of ESCs in a dose-dependent manner [3]. High levels of Oct4 increased the malignant potential of mouse ESC derived tumours, while Oct4 inactivation induced regression of the malignant component. It must be kept in mind that these tumours have a limited developmental potency. They never show a seminoma or choriocarcinoma component, and are predominantly composed of teratoma, and possibly yolk sac tumour upon extensive *in vivo* growth [16]. Oct4, therefore, plays a significant role in the malignant behaviour of ESC cells.

In ES cells, Oct4 appears to regulate cell fate in a dosage-dependent fashion [17]. Using a conditional expression and repression System demonstrated that

the level of Oct4 activity specifies three distinct fates of ES cells: 1) a_2-fold increase in expression turns ES cells into primitive endoderm and mesoderm; 2) repression of Oct4 induces the formation of trophectoderm; and 3) only an optimal amount of Oct3/4 can sustain stem cell self-renewal. These results suggest that ES cells must possess a network of regulators to keep Oct4 expression at the optimal level to ensure pluripotency [4]. How many transcription factors are involved in the regulation of Oct4 expression? This was the question asked by several groups in light of the observation that Oct4 must be maintained in a narrow range of expression levels to ensure stem cell pluripotency. The discovery of Nanog offered a clear candidate for Oct4 regulation. Named after Tir Nan Og (the Land of the Young), Nanog was discovered based on its ability to sustain stem cell self-renewal in the absence of LIF [17]. Although it was originally believed that Nanog prevents ES cell from differentiation in the absence of LIF by repressing the expression of differentiation genes, a simple reporter assay demonstrated that Nanog possesses two potent transactivators [18 & 19], suggesting that Nanog could be an activator of Oct4 expression. Indeed, Nanog behaves as a strong activator of the Oct4 promoter, thus participating in the regulation of Oct4 expression in ES cells [18].

In the last decade, a number of methods have been found to induce pluripotency artificially in somatic cells, including somatic cell nuclear transfer (SCNT) and cell fusion. Much of the research in this area has been carried out with mice, but the ultimate goal of stem-cell scientists remains the production of patient-specific pluripotent cells and their use in treatment of disease. Because both SCNT and cell fusion have posed technical and ethical problems as methods of reprogramming somatic cells, Takahashi and Yamanaka's method of reprogramming by defined factors has been hailed as the 'holy grail' of stem cell research. This method circumvents many of the problems associated with both SCNT and cell fusion and is regarded as the method with the best potential for producing patient-specific pluripotent stem cells for use in regenerative medicine [11].

Since the initial discovery of mouse iPS cells by Takahashi and Yamanaka, research in this area has advanced at an astonishing rate. In just over a year, the technology used to re-programme mouse cells has been successfully extended to human cells, while some of the initial problems with mouse iPS cells, including tumourigenicity have been partly addressed. Nevertheless, extensive research is still required with mouse iPS cells before any potential therapeutic use of human iPS cells is realised. The cause of the low efficiency of iPS induction remains to be determined. In addition to gene activation by expression of transcription factors, epigenetic remodelling plays a key role in induction of cellular pluripotency. A greater understanding of this mechanism will be necessary to improve the efficiency of iPS cell generation. Moreover, retroviral transduction involves random integration into the genome and consequently poses a risk of mutagenesis. The future use of alternative gene delivery systems or small molecules, which can replace retroviral gene products, may circumvent this problem.

Also, if iPS cells are to be used clinically, methods to direct differentiation and integrate them into tissues are still required. Despite this, however, iPS cells represent one of the best hopes for producing patient-specific stem cells for cell-based therapies.

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