Cloning of c-Myc 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 by reverse transcription-polymerase chain reaction (RT-PCR). Then c-Myc gene was amplified by (PCR) and inserted into the pTZ57R/T vector. Ligated product was 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. The cloned c-Myc gene can be used to prepare a gene cassette to produce stem cells from somatic cell.

Keywords: Cloning; Embryonic stem cells; c-Myc gene

INTRODUCTION

Human ES (Embryonic Stem) 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.

One of these regulatory genes is Myc (c-Myc) which encodes for a transcription factor. It is believed that it regulates expression of 15% of all genes through binding on Enhancer Box sequences (E-boxes) and recruiting histone acetyltransferases (HATs). This means that in addition to its role as a classical transcription factor, Myc also functions to regulate global chromatin structure by regulating histone acetylation both in gene-rich regions and at sites far from any known gene [3]. It can also act as a transcriptional repressor. For example, by binding Miz-1 transcription factor and displacing the p300 co-activator, it inhibits expression of Miz-1 target genes. In addition, myc has a direct role in the control of DNA replication [4].

Members of its family include the c-Myc, N-Myc, and the L-Myc genes. Myc gene was first discovered in Burkitt's lymphoma patients. In Burkitt's lymphoma, cancer cells show chromosomal translocations, in which Chromosome 8 is frequently involved. Cloning the break point of the fusion chromosomes revealed a gene that was similar to myelocytomatosis viral oncogene (v-Myc). Thus, the new found cellular gene was named c-Myc.

Myc family of transcription factors contain bHLH/LZ (basic Helix-Loop-Helix/Leucine Zipper) domain. Myc protein, through its bHLH domain can bind to DNA, while the leucine zipper domain allows the dimerisation with its partner, Max, another bHLH transcription factor. The Myc oncoprotein dimerizes with its partner, Max, to bind DNA, activate transcription, and promote cell proliferation, as well as programmed cell death. Max also forms homodimers or heterodimers with its alternative partners, Mad and Mxi-1. These complexes behave as antagonists of Myc/Max through competition for

common DNA targets, and perhaps permit cellular differentiation [5].

Myc is activated upon various mitogenic signals such as Wnt, Shh and EGF (via the MAPK/ERK pathway). By modifying the expression of its target genes, Myc activation results in numerous biological effects. The first to be discovered was its capability to drive cell proliferation (upregulates cyclins, downregulates p21), but it also plays a very important role in regulating cell growth (upregulates ribosomal RNA and proteins), apoptosis (downregulates Bcl-2), differentiation and stem cell self-renewal. Myc is a very strong proto-oncogene and it is very often found to be upregulated in many types of cancers. Myc overexpression stimulates gene amplification [6], presumably through DNA over-replication. The Myc proto-oncoprotein coordinates a number of normal physiological processes necessary for growth and expansion of somatic cells by controlling the expression of numerous target genes. Deregulation of Myc as a consequence of carcinogenic events enforces cells to undergo a transition to a hyper proliferative state. This increases the risk of additional oncogenic mutations that in turn can result in further tumor progression. However, Myc activation also provokes intrinsic tumor suppressor mechanisms including apoptosis, cellular senescence and DNA damage responses that act as barriers for tumor development. Myc thus possesses two seemingly contradictory "faces" referred to as "Yin and Yang"[7].

While endogenous Myc (c-myc) and Mycn (N-myc) have been reported to be separately dispensable for murine embryonic stem cell (mESC) function, myc greatly enhances induced pluripotent stem (iPS) cell formation and overexpressed c-myc confers LIF-independence upon mESC. To address the role of myc genes in ESC and in pluripotency generally, they were conditionally knocked out for both c- and N-myc using myc doubly homozygously floxed mESC lines (cDKO). Both lines of myc cDKO mESC exhibited severely disrupted self-renewal, pluripotency, and survival along with enhanced differentiation. Chimeric embryos injected with DKO mESC most often completely failed to develop or in rare cases survived but with severe defects. The essential nature of myc for self-renewal and pluripotency is at least in part mediated through orchestrating pluripotency-related cell cycle and metabolic programs. Endogenous myc genes are essential for mESC pluripotency and self-renewal as well as providing the first evidence that myc genes are required for early embryogenesis, suggesting potential mechanisms of myc contribution to iPS cell formation [8].

The object of this study is to clone human c-Myc gene in order to be used in converting somatic cells into stem cells.

MATERIALS AND METHODS

Cell culture

In this study Human embryonic stem line, (hSCT1; Stem cell technology research center, 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. 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 c-Myc gene

Total RNA of cells was extracted by RNAfast 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 using. For

accomplishment of reverse transcription (RT) reaction to obtain equivalent cDNA, we used expand 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 c-Myc gene related to Human embryonic stem cell was extracted from GenBank. Then primer designing was performed by oligo (Table 1). The primers were used to amplify and cloning of the complete sequence of c-Myc gene.

Table 1. The oligonucleotide primers used in the PCR assay

Primers	Sequence
Forward:	5- ATGCCCCTCAACGTTAGCTTCA -3
Reverse:	5- TTCCTTACTTTTCCTTACGCACA -3

The annealing temperature for primers was set at 52°C. The extension time was 60 seconds. B actin gene in all reactions was used as internal control. After the end of reaction the amount of 7 µl of the product was used to be run on 1% agarose gel electrophoresis and then staining results were compared with marker 1kbp (Fermentase). After all proprietary products, PCR product was purified and ligated into the cloning vector pTZ57R/T which then transformed into E. coli DH5α. On a medium containing antibiotic ampicillin, transformed bacteria were screened. PCR with specific primers was performed for some of the cloned hosts. The recombinant Colonies in PCR reaction was sent for sequencing.

RESULTS

Cell culture result

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

Gene cloning result

Proliferation area which code human c-Myc gene was provided in the presence of desired primers and 1320 bp PCR product of human c-Myc gene was obtained.

PCR Product after purification, was cloned in vector pTZ57R/T. PCR reaction of the plasmid on selective culture indicates some of these positive plasmids specific for the c-Myc primers. Results of the sequencing on plasmids extracted from positive plasmids (figure 2) and comparison with gene data bank confirmed cloning of this gene.

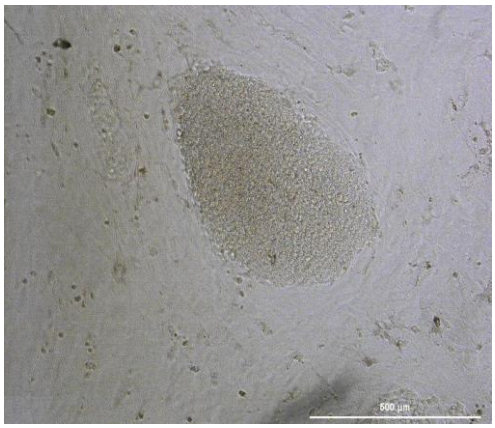

Figure 1. Human Embryonic stem cell line.

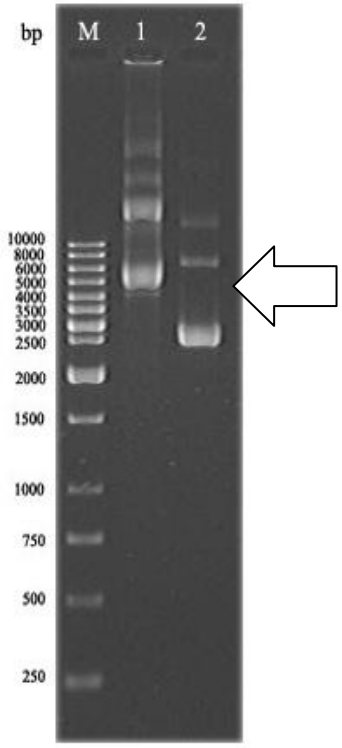

Figure 2. Plasmids extracted from hosts .Lane M: DNA molecular weight marker, Lane 1: Recombinant plasmid. Lane 2: Non recombinant plasmid.

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 pluripotency 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 reprogram 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 c-Myc seems to play a major role [9]. Dalton's group demonstrated an essential role for c-myc in normal LIF signalling in mESCs and showed that enforced c-myc expression conferred LIF-independent ESC growth [10], sparking great interest in myc function specifically in ESCs. Because Dalton had implicated myc in ESC function, the Yamanaka lab included myc in the pool of 24 candidates. Progress toward answering some key questions about myc function in iPS cell formation has now been reported in additional papers from the Yamanaka and Jaenisch groups. These studies report iPS cell production with a three-factor cocktail, that does not include myc, is also possible [11,12]. The efficiency of the iPS cell process without myc, is dramatically reduced and appears to progress much more slowly. Indeed, under standard antibiotic selection, the iPS cell process failed without myc. In half of the experiments conducted over an extended period of selection, no iPS cell colonies at all were produced when myc was excluded from the reprogramming protocol. An example of

successful iPS cell formation without myc, from adult tail tip fibroblasts, nonetheless yielded iPS cells with an almost 500-fold reduced efficiency, demonstrating that, at least under these assay conditions, myc fulfils an important role in the direct reprogramming process [11]. Thus, the ability to routinely and efficiently generate iPS cells may be myc dependent. Of great importance is the observation that mice generated from these three-factor iPS cells did not develop any tumours within 100 days [11], indicating that not all iPS cell-derived mice are prone to tumours.

But how could a proto-oncogene contribute to pluripotency or self-renewal of ESCs? Three models are proposed: (1) inducing a cell-cycle program necessary specifically for self-renewal, (2) modifying epigenetic patterns to promote dedifferentiation or block additional differentiation, and (3) selection of a rare population of cells with predetermined traits suited to permit induced pluripotency and self-renewal [13]. According to first item, myc may induce a stem-like cell-cycle program in fibroblasts whereby myc represses CDKI and stimulates D cyclins. The unique cell-cycle regulatory pathway in ESCs may be explained by a combination of low CDKI levels, high Cdk2 activity, and Rb hyperphosphorylation together maintained by elevated myc [10]. Also importantly, c-myc, albeit exogenous, sustained self-renewal in ESCs without strongly impinging on the cell cycle and was postulated to function by blocking differentiation [10]. According to the second item, myc contributes to iPS cell formation by inducing chromatin changes required for pluripotency [14] and according to third, it is also possible that among millions of fibroblasts lurk rare stem-like cells that are not fully differentiated and represent the only target cells amenable to direct reprogramming into iPS cell clones.

In the context of emerging regenerative medicine, myc has the potential to play a dual role in stem cells [13]. Levels of myc that are too high are likely to trigger apoptosis [10], whereas very low expression would inhibit induction of pluripotency. Thus the key to safely utilizing stem cells in regenerative medicine may reside in maintaining the appropriate expression levels, neither too high nor too low, of myc.

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 [9].

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 reprogram 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.

With regard to the previous studies, we can conclude that c-Myc gene has a central role in maintaining pluripotency and self-renewing properties of iPS cells. Thus, it can be considered as one of the essential candidates in somatic cells reprogramming which is applicable in cell transplantation and treatment of diseases. Therefore, in this study, we cloned this gene to be used later along with other essential gene candidates in making a gene cassette to produce iPS cells from somatic cells.

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