Expression of BMP4 mature peptide in eukaryotic cells and its differentiation-inhibiting effect in culturing induced pluripotent stem cells

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Abstract: Objective To investigate the role of bone morphogenetic protein 4 (BMP4) in culturing induced pluripotent stem cells (iPSCs) and the related signal pathways. Methods We amplified the mature peptide of BMP4 from the placenta through RT-PCR, and IgK secretion peptide was ligated to the N-terminal of BMP4 mature peptide. The recombinant plasmid pPYCAG-IgK-BMP4 was transfected into 293T cells and screened with puromycin, and the positive clones for expressing BMP4 were verified by cell immunofluorescence and Western blotting. To test the bioactivity of BMP4, iPSCs were cultured in the medium supplemented with leukemia inhibitory factor (LIF) plus the supernatant containing BMP4, and the cell phenotype, cell differentiation capacity into lineages of the 3 germ layers and expression levels of pluripotency-associated genes were investigated. Results Smad1 was phosphorylated by BMP4 from the culture medium. iPSCs cultured in the medium supplemented with LIF plus the supernatant containing BMP4 for 3 passages maintained the phenotype of stem cells with the expression levels of pluripotency-associated genes not affected. These iPSCs also maintained the capacity to differentiate into cell lineages of the 3 germ layers. Conclusion BMP4 can be efficiently expressed in mammalian cells to maintain the multipotent differentiation capacity of the iPSCs in in vitro culture.

Key words: bone morphogenetic protein 4; immunoglobulin kappa chains; over-expression; induced pluripotent stem cells; cell differentiation

INTRODUCTION

Embryonic stem (ES) cells with self-renewal and multipotent differentiation capacities can be isolated from the inner cell mass (ICM) of an embryo and cultured in vitro [1]. As a member of the transforming growth factor-β (TGF-β) family, bone morphogenetic protein 4 (BMP4) plays pivotal roles in the modulation of the self-renewal and proliferative activities of stem cells [2], and can induce human ES cells to differentiate into the trophoblast lineage in the presence of fibroblast growth factor (FGF) [3] and the expression of trophoblast-associated genes in the ES cells without FGF [4]. BMP4 is also capable of inducing the differentiation of mesendoderm in cooperation with activin [5]. All these evidences suggest that BMP4 importantly participates in the differentiation of human ES cells.

BMP4 assumes different roles in murine ES cell cultures. The differentiation of murine ES cells into neural lineage can be inhibited by treatment with BMP4 [6]. BMP4 in collaboration with leukemia inhibitory factor (LIF) sustains the self-renewal capacity of murine ES cells [7]. Further studies also confirmed the effect of BMP4 on murine ES self-renewal [8].

Both ES cells and induced pluripotent stem cells (iPSCs) are pluripotent stem cells derived from different tissues by different methods. iPSCs are commonly obtained by combinatorial expression of Oct3/4, Sox2, e-Myc and Klf4 transcription factors in embryonic fibroblast cells [9-11], and they share similarities to ES cells in gene expression profiles and differentiation potentials [12-14]. Genetically, iPSCs appear indistinguishable from ES cells, but they are not truly equivalent in comparative genomic analyses [15-16].

In this study, we aimed to express BMP4 mature peptide and analyze the bioactivity of BMP4 in iPSC culture and explore the pathways involved in the action of BMP4.
MATERIALS AND METHODS

Cell line and culture

Human 293T17 cell line was obtained from American Type Culture Collection (ATCC) and maintained routinely in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS, Biowest). Mouse iPSCs were produced in our lab previously in ES medium prepared with DMEM containing 10% FBS (Gibco), 1×NEAA, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 0.1 mmol/L 2-mercaptoethanol, supplemented with 10 ng/ml LIF. The iPSCs were cultured in NBM prepared with DMEM containing N2B27 (Gibco), 1×NEAA, 1 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 0.1 mmol/L 2-mercaptoethanol, supplemented with 10 ng/ml LIF and with 2% 1000 diluted supernatant of transfected 293T cells or not (BMP4+ or BMP4- NBM).

Immunofluorescence assay

The cells were fixed with 4% paraformaldehyde, washed in PBS for 3 times, and permeabilized with 0.1% Triton X-100 for 30 min. After washing with PBS for 3 times, the cells were blocked with 1% bovine serum albumin (BSA) and then incubated with 1: 200 BMP4 primary antibody (Chemicon, MAB4348) for 1 h at room temperature. After washing with PBS for 3 times, the cells were incubated with the secondary antibody for 30 min at room temperature, followed by staining of the cell nuclei with 10 ng/ml DAPI.

Differentiation of iPSCs

The iPSCs were cultured in BMP4- NBM for 3 passages. Without LIF and BMP4, the cells could form embryoid bodies in noncoated plastic dishes in a week. The embryoid bodies were attached to the dish bottom and began to differentiate. After 4 days, the cells were examined by immunostaining for expressions of Col2a1 (sc-52658, USA), a-fetoprotein (Glostrup, Denmark), and vimentin (ab20346).

Western blotting

The iPSCs were lysed with the lysis buffer (Beyotime, P0013B) containing PMSF. To determine the phosphorylation level of Smad1, the cells were stimulated by the supernatants of transfected 293T cells for 15 min. Anti-Oct4 (SC-5279, USA), anti-Sox2 (SC-17320, USA) and P-smad1/5/8 (#9511, CST) antibodies were used.

Semi-quantitative PCR of marker genes in iPS cells

Reverse transcription of the total RNA (2 μg) was performed with RT Reagent Kit (Takara code DRR037A). PCR was performed with ExTaQ (Takara code DRR041A). After amplification, 5 μl of the product from each reaction was loaded onto 2% agarose gel containing ethidium bromide (0.5 μg/ml). The sequences of the primers are listed in Tab.2. The experiments were repeated for 3 times.

RESULTS

Cloning BMP4 mature peptide from the mouse placenta

The signal peptide and leader peptide from BMP4 were replaced by Igk signal peptide (Fig.1A-B). The length of BMP4 was 348 bp (Fig.1C). Igk-BMP4 was inserted between XhoI and NotI restriction sites of pPYCAG plasmid (Fig.1D).

Expression of pPYCAG-Igk-BMP4 plasmid and bioactivity of BMP4

BMP4 was expressed in the cytoplasm of 293T

<p>| Tab.1 Primers for amplifying IgK-BMP4 |</p>
<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’-3’)</th>
<th>Round</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP4-1F</td>
<td>ATGAAGACCTACCCACAGCGGTCCAGG</td>
<td>First round</td>
</tr>
<tr>
<td>BMP4-1R</td>
<td>AATCGCGCCGTCAGCCATCCACACCCCTCTAC</td>
<td></td>
</tr>
<tr>
<td>IgK-BMP4-2F</td>
<td>CTGCTGCTTGGGTCTGGTCACTGGTGACATGAGCAGCATCACCCA</td>
<td>Second round</td>
</tr>
<tr>
<td>IgK-BMP4-2R</td>
<td>AATCGCGCCGTCAGCCATCCACACACCCCTCTAC</td>
<td></td>
</tr>
<tr>
<td>IgK-BMP4-3F</td>
<td>GGACTCGAGATGGAGACAGACACACTCCTCTGAATGGGTGCTGCTGTC</td>
<td>Third round</td>
</tr>
<tr>
<td>IgK-BMP4-3R</td>
<td>AATCGCGCCGTCAGCCATCCACACACCCCTCTAC</td>
<td></td>
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cells transduced with pPYCAG-IgK-BMP4, but not detected in cells transduced with pPYCAG (Fig.2A). Quantitative analysis of the supernatant by Western blotting demonstrated BMP4 release in the medium by pPYCAG-IgK-BMP4-transfected cells (Fig.2B). No significant variations were observed in the expression levels of BMP4 in the cytoplasm at different time points, nor in BMP4 levels in the supernatant (Fig.2C). Smad1 was phosphorylated by BMP4 in the supernatant of pPYCAG-IgK-BMP4-transfected cells within 15 min (Fig.2D).

Self-renewal of iPSCs

iPSCs were maintained in BMP4+ or BMP4 NBM and passaged for three times. LIF combined with BMP4 in the medium efficiently prevented iPSC differentiation (Fig.3A and B), but in the absence of BMP4, LIF alone failed to maintain the phenotype of stem cells (Fig.3C). The cells cultured in BMP4+ NBM did not exhibit significant changes in Sox2/Oct4 protein expressions compared to the cells cultured in ESM. In BMP4- NBM, the cells showed down-regulated Sox2/Oct4 expression compared to the cells cultured in ESM or in BMP4+ NBM (Fig.3D). The mRNA expression levels of other pluripotency-associated genes also showed changes similar to those of Sox2/Oct4 protein expression (Fig.3E).

iPSC differentiation into three germ layers

iPSCs still maintained the differentiation potential after culturing for 3 passages in BMP4+ NBM and expressed the marker genes (AFP, Col2a1 and vimentin) of the three germ layers (Fig.4).

DISCUSSION

Numerous evidences have been obtained concerning the transcriptional networks that regulate ES cells, but currently little is known of the signaling pathways that control the reprogramming and maintain the pluripotent state of the cells. A recent study showed that BMP signaling promotes reprogramming during the initiation stage, and the effect of BMPs on reprogramming depends on the presence of Oct3/4, Sox2, c-Myc and Klf4. BMP4 also greatly improves the efficiency of Oct4/Sox2 or Oct4 reprogramming. The role of BMP4
in culturing iPSCs is still unknown. In the present study, the effects of the supernatant containing BMP4 on iPSCs were analyzed.

Several measures were taken to obtain higher expressions of BMP4. A highly efficient eukaryotic expression system exploiting the episomal vector pPYCAG and 293T cell line was used. pPYCAG expression vector contains the hybrid promoter CAG which is a combination of the cytomegalovirus (CMV), early enhancer element and chicken beta-actin promoter. Episomal vector can be replicated continuously after transfection into cells that contain a large T antigen such as 293T cells[19-20]. Such replication allows for maintenance of stable expression of the recombinant protein. The expression of a recombinant protein is also associated with its gene length. BMP4 gene is constituted by 3 elements, namely the signal peptide, leader peptide, and mature peptide, and the mature peptide executes the major function of BMP4 [21]. We amplified the mature peptide of BMP4 by RT-PCR from mouse placenta. To express BMP4 in the form of secretion in the supernatant, the coding sequences for a mouse immunoglobulin k chain (Igk) signal peptide was fused to the N-terminal of BMP4 mature peptide. This signal peptide also increased the expression level of BMP4 in the supernatant.

Cell immunofluorescence assay was employed to verify the expression of BMP4 in 293T cells. Compare to the control cells, the transfected cells showed obvious

Fig.2 Expression of BMP4 in 293T17 cells after stable transfection with the episomal vector. A: Immunofluorescence analysis of BMP4 expression in 293T cells (×200); B: BMP4 (protein molecular weight 18 000) from the supernatant 14 days post-transfection detected by Western blotting; C: BMP4 expression in the cytoplasm and supernatant at different time points post-transfection; D: BMP4 from the supernatant activates Smad1 in comparison with the control supernatant.
BMP4 expression in the cytoplasm. To eliminate the trace of BMP4 in FBS, 293T cells screened by puromycin were cultured in the medium in the absence of LIF and BMP4 before the supernatants were collected. Thus, the proteins present in medium were totally cell-secreted proteins. Western blotting showed that BMP4 was released in the medium 14 days after the transfection. We also found that the expression levels of BMP4 were stable in both the cytoplasm and supernatant 2, 4, 6, and 8 weeks after the transfection, which further confirmed the correct construction of pPYCAG-Igk-BMP4 vector and the stable expression of BMP4 in 293T cells.

iPSCs, like ES cells, also have self-renewal and differentiation potentials. iPSCs exhibits the phenotype of ES cells when cultured in ESM. Obvious differentiated cells were observed around the clone when ESM was replaced by BMP4-NBM. The cell differentiation was inhibited efficiently by BMP4-NBM. The expression levels of pluripotency-associated genes in iPSCs cultured in different medium were consistent with the cell phenotypes. Differentiation of iPSCs into the three germ layers in vitro indicates that BMP4 supports the self-renewal of iPSCs in the presence of LIF.

BMPs including BMP4 had proved to be essential for the reprogramming of somatic cells. Our findings confirmed that BMP4 was indispensable for culturing iPSCs. However, Smad1 was activated by the supernatant containing BMP4. This is a new finding for iPSCs, suggesting that iPSCs may share the same pathway of self-renewal that ES cells exploit.

Acknowledgments

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References
真核表达BMP4成熟肽及其对iPS细胞的分化抑制作用

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摘要: 目的 研究BMP4因子在诱导型干细胞(iPS细胞)培养过程中的作用和后期作用的相关途径。方法 通过RT-PCR的方法从小鼠的胎盘组织中扩增BMP4成熟肽, 并且在其N末端连接IgK分泌肽, 此融合的片段克隆至pPYCAG载体上。重组质粒pPYCAG-IgK-BMP4转染至293T细胞中并进行嘌呤霉素的筛选。通过细胞免疫荧光和Western blot方法鉴定出表达BMP4的阳性克隆。结果 Smad1和Smad4的阳性克隆中, 稳定表达BMP4的细胞培养上清和LIF因子中, 在BMP4-2M5和5M5的效验电泳方法及BMP4的阳性克隆中, 稳定表达BMP4的细胞培养上清和LIF因子中, 可以有效抑制iPS细胞的分化。在BMP4-2M5和5M5的效验电泳方法及BMP4的阳性克隆中, 稳定表达BMP4的细胞培养中, 通过细胞免疫荧光和Western blot方法鉴定出表达BMP4的阳性克隆。结论 BMP4因子在诱导型干细胞(iPS细胞)培养过程中的作用和后期作用的相关途径。