Efficient gene transfer to endometrial adenocarcinoma cell line (Ishikawa) by To12 transposable element: a possible DNA vector for gene therapy for implantation failure

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Abstract

In spite of the progress of reproductive assistance by means of infertility therapy, the treatment of implantation failure caused by the lack of cytokine genes is very difficult at the present time, therefore it is hoped that the development of new technology of gene transfection to human endometrium is overcome this problem. In this study we examined whether the To12 transposable element, a DNA-type transposon identified from the Japanese medaka fish, can efficiently introduce a foreign DNA into the genome of the endometrial adenocarcinoma cell line Ishikawa, the best available model of human endometrium, as a preclinical study on gene therapy for implantation failure. The To12 transposable element in this study consisted of a transposon-donor plasmid DNA containing a To12 transposon construct, carrying the neomycin-resistance gene and a helper plasmid DNA that can produce the transposase protein. In this study, we found that the number of neomycin-resistant colonies increased four fold when the transposon-donor plasmid was co-transfected with the helper plasmid, compared with when the donor-plasmid was transfected without the helper plasmid. We analyzed the transposon integration sites and found that the To12 construct was indeed integrated into the genome of Ishikawa cells through transposition. Our study revealed that the To12 transposon system can function in Ishikawa cells and create chromosomal integration of a foreign DNA very efficiently, expressing the gene on the integrated To12 vector. The To12 transposon system should be applicable to delivery of cytokine genes to the human endometrium, which may lead to a treatment for implantation failure. Moreover it may be possible to apply the transposon transfection system clinically not only to the treatment of implantation failure infertility, but to diseases caused by abnormalities in a variety of genes, including cancer and may have applications in regenerative medicine in the future.

Introduction

Reproductive assistance by means of infertility therapy, especially in vitro fertilization and embryo transfer (IVF–ET), has advanced remarkably in recent times. However, the pregnancy rates of IVF–ET are still low, and, in many cases, implantation is blocked despite the transfer of good quality embryos⁴. Recently, a variety of cytokines have been found to contribute locally in the endometrium during the implantation window, and the lack of these cytokines has been considered to cause implantation failure in experimental systems using knockout mice⁵. Based on these findings, supplying cytokines to the endometrium may have applications in the future.
the endometrium may be a means to improve implantation failure. To enhance the expression of such cytokines in the endometrium, it is necessary to transfer the cytokine genes into the endometrium efficiently. Experimental attempts have been made to transfer genes of a variety of cytokines into the endometrium of mice using viral vectors. Although the success of transfer of foreign genes into endometrial cells has been reported\(^8\), it has also been reported that the expression of the foreign gene in the endometrial cells was temporary and declined rapidly\(^9\).

Other than using retroviral vectors, attempts to conduct gene transfer in mammalian cells by using a transposon vector have been made\(^8\)\(^-\)\(^11\). In comparison to gene transfer by viral vectors, a nonviral gene transfer system such as a transposon system may have the following advantages. First, it is easier and safer to manipulate and prepare transposon vectors, whereas manipulation and preparation of most viral vectors are laborious and costly. Second, gene transfer by a transposon vector does not have the risks related to pathogenicity, which may be caused by the virus itself or by host immune reactions against viral infection. Third, although it is known that gene expression from infected viruses often does not last for a long time\(^6\)\(^-\)\(^8\), it is anticipated that gene expression of a foreign gene from an integrated transposon vector will persist for a longer period of time\(^11\)\(^-\)\(^13\).

The To12 element, a DNA type transposon identified from the Japanese medaka fish, is an active transposable element that encodes a gene for a functional transposase\(^14\)\(^-\)\(^17\). Gene transfer systems using the To12 element have been developed and described in several reports on vertebrates\(^9\)\(^17\)\(^18\). In mammals, the To12 transposon system has been applied to introduce a foreign gene into the genome of mouse embryonic stem (ES) cells\(^9\). This system consists of two components; a transposon–donor plasmid and a helper plasmid. The transposon–donor plasmid contains a To12 transposon construct carrying the neomycin–resistance gene under the control of the PGK promoter, and the helper plasmid contains the transposase cDNA under the control of the CAG promoter\(^8\). When these plasmids were introduced into mouse ES cells by electroporation, the transposase protein was produced from the helper plasmid, and the transposon portion was excised from the transposon–donor plasmid and integrated into the genome. The chromosomal integration of the transposon construct resulted in expression of the neomycin–resistance gene\(^8\).

In this study, we sought to determine whether the To12 transposable element can act as a vector to transfer a foreign gene to the human–derived Ishikawa cell line, the best available model of human endometrium.

### Materials and Methods

#### Transfection to Ishikawa cells

The structures of the three kinds of plasmids (pT2KP-Kneo plasmid, pCAGGS-T2TP plasmid, pCAGGS plasmid) used in this study are shown in Figure 1\(^9\). The human endometrial adenocarcinoma cell line, Ishikawa cells\(^9\)\(^20\), was donated by Dr. M. Nishida (National Kasumigaura Hospital). Ishikawa cells were cultured in modified Eagle’s medium (MEM) containing 5% (vol/vol) non-stripped fetal calf serum (FCS), 200 nM L-glutamine, and 5% gentamycin. The cells were grown in 10-cm dishes in a humidified chamber containing 95% air/5% CO\(_2\) at 37°C. When cells were nearing confluence, they were prepared for lipofection, as described by Felgner et al and Hawley–Nelson et al\(^21\)\(^-\)\(^22\). In each transfection experiment, \(2 \times 10^6\) Ishikawa cells were transfected with pT2KP-Kneo (5 μg) and pCAGGS–T2TP (5 μg) or pT2KP-Kneo (5 μg) and pCAGGS (5 μg), and incubated for two days. The cells were then removed from the dishes, re-seeded onto ten 10-cm culture dishes, and cultured on MEM medium containing G418 (1 mg/ml) and 5% FCS. Twelve days after re-seeding, the number of G418-resistant colonies on the 10-cm dishes was counted using a microscope. Further, 12 colonies from each transfection group, the pT2KP-Kneo and pCAGGS–T2TP–transfected group and the pT2KP-Kneo and pCAGGS–transfected group, were isolated and cultured separately on 10-cm dishes in the presence of G418 until they reached confluence.

#### Southern blot hybridization analysis

Genomic DNA was extracted from Ishikawa cells of each of the 12 clones obtained from the pT2KP-Kneo and pCAGGS–T2TP–transfected group and the pT2KP-Kneo and pCAGGS–transfected group. Southern blot hybridization was performed as described by Kawakami et al\(^9\). The genomic DNA was digested with BglII, separated on 1% agarose gel, transferred to a nylon membrane, and hybridized with the 32P-labeled neo gene probe (Figure 1).

#### Analysis of To12 integration sites by linker-mediated polymerase chain reaction (PCR)

To clone and sequence the junction fragments of the integrated To12 and genomic DNA, we performed linker-mediated PCR\(^23\)\(^-\)\(^24\)\(^-\) with some modifications. The genomic DNA from the Ishikawa clones was digested with MboI, BglII, BamHI, SpeI, XbaI, or Nhel. MboI, BglII and BamHI generate a 5′-GATC-3′ cohesive end. SpeI, XbaI and Nhel generate a 5′-CTAG-3′ cohesive end. The GATC adaptor or the CTAG adaptor was prepared by annealing AL (5′-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG GAG GT-3′) with phosphorylated GATC–AS (5′-pGAT CAC CTG CCC CCG CTT-3′) or phosphorylated CTAG–AS (5′-
Fig. 1 Structure of *To12* transposable element and plasmids used in this study.
Thick red lines indicate the full-length *To12* element, and the thin blue line on the top indicates transcription of the transposase gene encoded by *To12*. The arrow and broken lines in the transcript show the direction of the transcript and introns, respectively. *pT2KP*Kneo: the transposon donor plasmid containing the *To12* construct with the neomycin expression cassette. The *Bgl*II sites used for Southern blot hybridization analysis are shown. The solid black bar above *pT2KP*Kneo indicates the probe used for Southern blot hybridization. *pCAGGS-T2TP*: the helper plasmid used to express the transposase protein in transfected Ishikawa cells.

![Diagram of *To12* transposable element and plasmids](image)

**Fig. 2** Transposition in cultured Ishikawa cells.
Left: Ishikawa cells were transfected with the *pT2KP*Kneo plasmid and the *pCAGGS* plasmid. Right: Ishikawa cells were transfected with the *pT2KP*Kneo plasmid and the *pCAGGS-T2TP* plasmid. For both of them, 14 days after transfection, cells were stained with methylene blue, and the number of colonies was counted. The average number of G-418-resistant colonies was obtained from 10 dishes for two independent transfections.

pCTA GAC CTG CCC CCG CTT-3') at 95°C for 1 min, respectively. Then the GATC or the CTAG adaptor was ligated to the genomic DNA digested with *MboI*, *Bgl*II, and *BamH*I or with *Spe*I, *Xba*I and *Nhe*I, respectively. The ligated samples were diluted 10-fold, and used for PCR. To clone 5' junctions, the first PCR was performed using primers: Ap1 (5'-GGA TCC TAA TAC GAC TCA CTA TAG GG-3') and 175L-out (5'-TTT TTG ACT GTA AAA ATT G-3'), and the second PCR was performed using primers: Ap2 (5'-CAC TAT AGG GCT CGA GCG G-3') and 150R-out (5'-GAG TAA AAA GTA CTT TTT TTT CTT CT-3'). To clone 3' junctions, the first PCR was performed using primers: Ap1 and 175R-out (5'-TTC TTG CTT TTA CTT TTA CTT CC-3'), and the second PCR was performed using primers: Ap2 and 150R-out (5'-AAT ACT CAA GTA CAA TTT TA-3'). The PCR products for the 5' and 3' junctions were gel-extracted and sequenced using 100L-out (5'-AGT ATT GAT TTT TAA TTG TA-3') and 100R-out (5'-AGA TTC TAG CCA GAT ACT-3').
3'), respectively.

Results

Transfection of the Tol2 vector carrying neomycin resistance gene to Ishikawa cells

In order to test whether the Tol2 transposon system can generate chromosomal integration of a foreign gene in human endometrium cells, Ishikawa cells were co-transfected with pT2KPKneo, the transposon–donor plasmid containing the Tol2 vector with the neomycin resistance gene, and pCAGGS–T2TP, the helper plasmid which was used to express the transposase protein in mouse ES cells, by lipofection. Also, as a control, Ishikawa cells were co-transfected with pT2KPKneo and pCAGGS, the vector plasmid without the transposase gene. The transfected cells were divided into ten 10-cm dishes and cultured in the presence of G418 as described in Materials and Methods. Fourteen days after transfection, G418-resistant colonies were formed in both transfection experiments, and the number of colonies on the 10 dishes for each transfection experiment was counted. On average, 22,800±1,050 G418-resistant colonies were formed on dishes transfected with pT2KPKneo and pCAGGS–T2TP, while 4,900±1,248 G418-resistant colonies were formed on dishes transfected with pT2KPKneo and pCAGGS (Figure 2). Thus, the number of G418-resistant colonies was about four-fold greater with transfection with pT2KPKneo and pCAGGS–T2TP than with pT2KPKneo and pCAGGS. This result indicates that the transposase activity supplied by pCAGGS–T2TP enhanced the integration efficiency in Ishikawa cells.

Southern blot hybridization analysis of G418-resistant clones

Twelve G418-resistant colonies from each transfection experiment were picked up and expanded on 10-cm dishes. We thus established 12 G418-resistant clones for each transfection condition. Genomic DNA was prepared from these cell clones, and analysed by Southern blot hybridization. The results are shown in Figure 3. In the cell clones transfected with pT2KPKneo and pCAGGS–T2TP, 1 (Figure 3, lane 16) to 10 (Figure 3, lane 20) bands were detected, and, in total 52 bands were observed in the 12 clones. On the other hand, in the cell clones transfected with pT2KPKneo and pCAGGS, one (Figure 3, lanes 2, 4, 10 and 12) to four (Figure 3, lane 5) bands were detected, and in total, 22 bands were observed in the 12 clones. These results indicated that: 1) in both transfection experiments the T2KPKneo sequence was integrated into the genome of Ishikawa cells, 2) the increase in number of insertions in cells transfected with pT2KPKneo and pCAGGS–T2TP indicated that the transposase activity enhanced the chromosomal integration, and 3) in both transfection experiments the results indicated that a single copy of the neomycin resistance gene could confer G418-resistance to Ishikawa cells.

Identification of integration sites

To determine whether the insertions of the T2KPKneo DNA were indeed created through transposition, we characterized the integration sites at the sequence level. We randomly selected two clones (lane 14 and lane 15 in Figure 3) out of the 12 G418-resistant clones obtained by co-transfection using pT2KPKneo and pCAGGS–T2TP, extracted genomic DNA from these two clones, and cloned the junctions of the integrated T2KPKneo transposon and the surrounding genomic DNA by the linker-mediated PCR method. As a result, we found that five 5' junction clones and five 3' junction clones contained the same 8-bp sequence adjacent to the T2KPKneo sequence. It is known that integration of Tol2 creates 8-bp direct repeats at both ends. It is likely that these five 5' and 3' junction clones were derived from five different transposon integrations. Further, in these five cases, research using Basic Local Alignment Search
Tool (BLAST) using the 5’ and 3’ junction sequences hit the same loci, respectively. From these results, we concluded that these sequences were derived from the 5’ and 3’ ends of single independent insertions, which corresponded to 2 bands on lane 14 and 3 bands on lane 15.

The results of the BLAST search using the sequences surrounding transposon insertions are summarized in Table 1. The integration sites were dispersed on different chromosomes, some within genes and some in intergenic regions. We did not find any sequence specificity at the target sites.

### Discussion

In this study, we demonstrated that the Tol2 transposon system can create chromosomal integration very efficiently in the Ishikawa human endometrial cell line. We showed that the transposon-donor plasmid, pT2KPKneo, alone was integrated into the genome of Ishikawa cells even in the absence of transposase activity, probably through non-homologous recombination between the plasmid DNA and the genomic DNA. However, when the transposase protein was supplied, the transposon portion was excised from the donor plasmid and integrated into the genome of Ishikawa cells by the catalytic activity of transposase. We showed that 8-bp direct repeats were created at the integration sites of the transposon vector, indicating that the insertions were indeed created through transposition. The transposition reaction gave rise to an about four-fold increase in the efficiency of chromosomal integration in our present transfection conditions. These findings suggest that it will be possible to use the Tol2 transposition system as an effective vector for gene therapy in human endometrial cells in the future.

The Sleeping Beauty transposon, a synthetic transposon constructed based on the sequences of transposons of the Tcl1/mariner family, has been used as a gene transfer vector in mammalian cells, and used to introduce the gene for blood coagulation factor IX into the liver cells of hemophilia mice in vivo. Chromosomal transposition resulted in long-term expression (>5 months) of human blood coagulation factor IX at levels that were therapeutically effective in hemophilia mice. Although these studies raised the possibility that the transposon system could be applied to human gene therapy, these techniques still require further development. The results of the present study suggest that the Tol2 transposon system, as well as the Sleeping Beauty transposon system, may be utilized for gene transfer in humans as a nonviral vector.

Gene transfer by means of transposons may have several advantages over the use of viral vectors. First, a nonviral gene transfer system is easy to produce and purify, and the procedure is not as laborious or costly. Second, the transposon system does not seem to have pathogenicity or induce an immune reaction by itself, and should be free from the risk of pathogenesis caused by by-products created through viral recombination. Third, it is easy to handle, and, once a transposon is integrated into the cells, it is stable and safe and we do not have to worry about random circulation. Thus, transposons are promising new tools for gene therapy in the future, and we propose that the Tol2 transposon system should be used for gene transfer into human endometrial cells.

Many attempts have been made to elucidate the cause of implantation failure. Although morphological abnormalities related to implantation failure have been frequently observed, little is known about the functional basis for the defects that lead to the morphological abnormalities. However, there has been a case where a defect in cytokine expression was thought to be the cause for formation of a thin endometrium at the time of the implantation window, which should be thickened in the presence of an adequate supply of necessary cytokines at that time. Consistent with this, knock-out of some cytokine genes in the mouse caused implantation defects. Therefore, it can be argued that some cases of implantation failure may be treated by enhancing cytokine expression locally in the endometrium.

Towards the future goal of such treatment by gene therapy, several attempts to introduce a foreign gene into endometrial cells have been made. Bagot et al. delivered a DNA/liposome complex into the uterine cavity of mice and observed expression of lacZ and hoxa10 in endometrial cells. Nakamura et al. succeeded in transferring foreign genes into the mouse uterus using a hemagglutinating virus of Japan envelope vector.

### Table 1. Cloning and sequencing analysis of junction fragments

<table>
<thead>
<tr>
<th>Tol2 ID</th>
<th>Target sequence</th>
<th>Chromosome</th>
<th>Putative features</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>CAATCTCAAGCTACTG</td>
<td>8</td>
<td>Alu repeat</td>
</tr>
<tr>
<td>2</td>
<td>ATGAGTTGAAGGGTC</td>
<td>1</td>
<td>Intron of CD84 antigen (leukocyte antigen)</td>
</tr>
<tr>
<td>3</td>
<td>TGGAGTTGGAGCACTC</td>
<td>8</td>
<td>Intergenic region -20kb from ENSG00000184078</td>
</tr>
<tr>
<td>4</td>
<td>GGCTGTCTTAACATT</td>
<td>18</td>
<td>Intron of enolase superfamily member 1 (ENOSF1)</td>
</tr>
<tr>
<td>5</td>
<td>ATTGTGTGTTGGGCTT</td>
<td>10</td>
<td>Intron of Loc55328</td>
</tr>
</tbody>
</table>

1. The 8-bp sequence duplicated upon insertion of the Tol2 element is underlined.
Our goal is to use the Tol2 transposon system in vivo to treat implantation failure in humans caused by decreased cytokine activity. For this purpose, the next step will be introduction of the Tol2 transposon construct and transposase activity into the mouse uterine cavity, and testing whether a foreign gene carried by the Tol2 vector can be expressed in endometrial cells. The present in vitro study is an important step towards this goal. We propose that the Tol2 transposon system offers several advantages over viral vectors and should be applied clinically not only for treatment of infertility due to implantation failure, but also for treatment of a variety of diseases caused by defects in gene function, including cancer therapy and regenerative medicine.

Acknowledgements

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References

トランスポソンによる子宮内膜腺癌株（イシカワ株）への効果的な遺伝子導入：着床障害不妊症の遺伝子治療に対する有益なDNAベクター

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生殖補助医療の進歩により、着床障害性不妊症の原因が子宮内膜に存在すべきサイトカイン遺伝子の欠如およびそれらの発現の低下によるものであることが示唆され、子宮内膜細胞内にこれらの遺伝子を遺伝子導入する試みがなされてきている。

今回我々は、メダカ由来のトランスポソンによる遺伝子導入システム（Tol2ベクター）がヒト由来細胞（子宮内膜上皮細胞のモデル細胞株であるイシカワ株）において有効に機能するかについて検討をおこなった。Tol2ベクターにネオマシン耐性遺伝子を組み込み、イシカワ株での遺伝子発現の有無を確認したところ、その発現が観察され、またTol2転移酵素存在下において非存在下の約4倍もの効率で遺伝子導入がなされていることが明らかとなった。さらに挿入されたTol2の両末端において8塩基対の反復配列が観察された。このことより、Tol2ベクターによる遺伝子導入システムはウイルスベクターに比べてより有効なベクターとして今後活用されることが期待される。

本研究は着床障害に対する遺伝子治療の第一歩であるが、今後遺伝子異常によって引き起こされる疾患・癌治療・再生医療を含めた幅広い医療に臨床応用されることが期待される。

キーワード 子宮内膜、遺伝子導入、着床障害、イシカワ株、トランスポソング