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Knockdown of gene expression by antisense morpholino oligos in preimplantation mouse embryos cultured *in vitro*

Yuki Sato [a], Shiori Sato [a], Takahiro Kikuchi [a], Asumi Nonaka [a], Yuki Kumagai [a], Akira Sasaki [a, b], Masayuki Kobayashi [a, *]

[a] Graduate School of Bioresource Sciences, Akita Prefectural University, 241-438 Shimoshinjho Nakano, Akita 010-0195, Japan
[b] Akita Research Institute of Food and Brewing, 4-26 Sanuki, Araya-machi, Akita 010-1623, Japan

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ABSTRACT

Knockdown of gene expression by antisense morpholino oligos (MOs) is a simple and effective method for analyzing the roles of genes in mammalian cells. Here, we demonstrate the efficient delivery of MOs by Endo-Porter (EP), a special transfection reagent for MOs, into preimplantation mouse embryos cultured *in vitro*. A fluorescein-labeled control MO was applied for monitoring the incorporation of MOs into developing 2-cell embryos in the presence of varying amounts of EP and bovine serum albumin. In optimized conditions, fluorescence was detected in 2-cell embryos within a 3-h incubation period. In order to analyze the validity of the optimized conditions, an antisense *Oct4* MO was applied for knockdown of the synthesis of OCT4 protein in developing embryos from the 2-cell stage. In blastocysts, the antisense *Oct4* MO induced a decrease in the amount in OCT4 protein to less than half. An almost complete absence of OCT4-positive cells and nearly complete disappearance of the inner cell mass in the outgrowths of blastocysts were also noted. These phenotypes corresponded with those of *Oct4*-deficient mouse embryos. Overall, we suggest that the delivery of MOs using EP is useful for the knockdown of gene expression in preimplantation mouse embryos cultured *in vitro*.

1. Introduction

Pluripotency-maintaining and differentiation-inducing genes are expressed during the preimplantation development of mouse embryos. In the early mouse embryo, the first irreversible segregation of cell fate occurs between the 8-cell stage and the morula stage [1]. At the 16-cell morula stage, the blastomeres enhance cell-cell contacts to become compacted morulae, and the subsequent cell-divisions increase the topological complexity of the morula to differentiate into two distinct cell phenotypes, such as the outside cell populations, trophectoderm, and the inside cell populations, the pluripotent inner cell mass (ICM) [2–4]. Cells comprising the ICM are capable of differentiating into all fetal and adult cell lineages. The roles of genes expressed during this period have been analyzed by the generation of targeted gene-knockout mice. For example, knockout of the *Oct4* gene led to embryonic lethality with the loss of ICM-derived cell lineages. *Oct4*-knockout zygotes developed *in vitro* to the blastocyst stage with morphologically normal ICM [5]. Thereafter, outgrowths of the blastocyst developing from *Oct4*-knockout zygotes were not able to maintain the ICM and preferentially differentiated into trophoblastic giant cells. Although the results obtained from the gene-knockout mice are generally clear, this technique is not easily used, because it is laborious, time-consuming, and expensive. On the other hand, gene knockdown technologies, such as those utilizing small interfering RNAs (siRNAs), have been also applied. Delivery of siRNAs into preimplantation embryos is difficult because embryos are surrounded by the zona pellucida. Therefore, a microinjection procedure, which requires technical skill and special equipment, such as a micromanipulator, is usually combined.

Morpholino oligos (MOs) provide a simple and effective gene-silencing method that enables inhibition of the function of a specific gene while minimizing side-effects seen in other antisense

Abbreviations: BSA, bovine serum albumin; DAPI, 4′-6-diamino-2-phenylindole; EP, Endo-Porter; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; hCG, human chorionic gonadotropin; ICM, inner cell mass; LIF, leukemia inhibitory factor; MO, morpholino oligo; PVDF, polyvinylidene fluoride; siRNA, small interfering RNA; WM, modified Whitten's medium.

* Corresponding author. Laboratory for Advanced Animal Cell Technology, Graduate School of Bioresource Sciences, Akita Prefectural University, 241-438 Shimoshinjho Nakano, Akita 010-0195, Japan.
E-mail address: makoba@akita-pu.ac.jp (M. Kobayashi).

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technologies, such as rapid degradation by nucleases [6]. Because MOs are nonionic, enhancement of transfection efficiency by lipofection reagents is not suitable. In this study, we applied Endo-Porter (EP), a peptidic transfection regent specifically developed for MOs [7], for the delivery of MOs into preimplantation mouse embryos cultured *in vitro*. In addition, we tried to phenocopy *Oct4*-knockout embryos in wild-type embryos by introducing an antisense *Oct4* MO using EP.

2. Materials and methods

2.1. Media

M2 medium [8] was supplemented with 4 mg/ml bovine serum albumin (BSA, #A4378, Sigma, St. Louis, MO, USA), and modified Whitten's medium (WM) [9] was supplemented with 3 mg/ml BSA. Phenol red was not added to M2 medium or WM. Glasgow modified Eagle's medium (Sigma) was supplemented with 10% fetal calf serum (FCS, defined for mouse ES cells, Biological Industries, Haemek, Israel), 10^{-4} M 2-mercaptoethanol (Nacalai Tesque, Kyoto, Japan), 1 mM sodium pyruvate (Sigma), 1 mM non-essential amino acids (Nacalai Tesque), and recombinant human leukemia inhibitory factor (LIF, prepared in-house [10]), and this was referred to as ES medium.

2.2. Embryo collection

To obtain *in vivo*-fertilized embryos, 6–12 weeks old virgin female CD-1 mice (random bred, Swiss, Charles River Japan, Tokyo, Japan) were housed in controlled illuminated conditions (lights on from 0700 to 1900 h) at 22 °C. The female mice were induced to superovulate by intraperitoneal injections of 10 IU equine chorionic gonadotropin (Zenoac, Koriyama, Japan) followed 48 h later by 10 IU human chorionic gonadotropin (hCG, Zenoac), and they were then mated overnight with fertile males (15–30 weeks old). Zygotes were flushed in M2 medium from excised oviducts 21–23 h after hCG injection. Cumulus cells were removed from the zygotes by hyaluronidase treatment (150 units/ml in M2 medium without BSA, Type I-S, Sigma). Two-cell embryos were flushed in M2 medium from excised oviducts 41–42 h after hCG injection. All animal procedures conformed to the Guidelines for the Care and Use of Laboratory Animals of Akita Prefectural University.

2.3. Culture of embryos

Flushed embryos were transferred into 100 μl drops of fresh WM supplemented with MOs in the presence of BSA (3 mg/ml [9] or 60 μg/ml [11]) and EP (#Endo-P, Gene Tools, Philomath, OR, USA) under mineral oil (Nacalai Tesque). When zygotes were cultured, EDTA (50 μM) was added because low concentrations (10–50 μM) of this compound have been demonstrated to promote the development of CD-1 zygotes cultured *in vitro* [12]. Fluorescein isothiocyanate (FITC)-labeled or non-labeled MOs (5′-CCT CCTACCTCAGTTACAATTTATA-3′, GeneTools) against human thalassemic *Hbb* (also known as β-globin) pre-mRNA were used as a control MO. It was reported by the manufacturer that the control

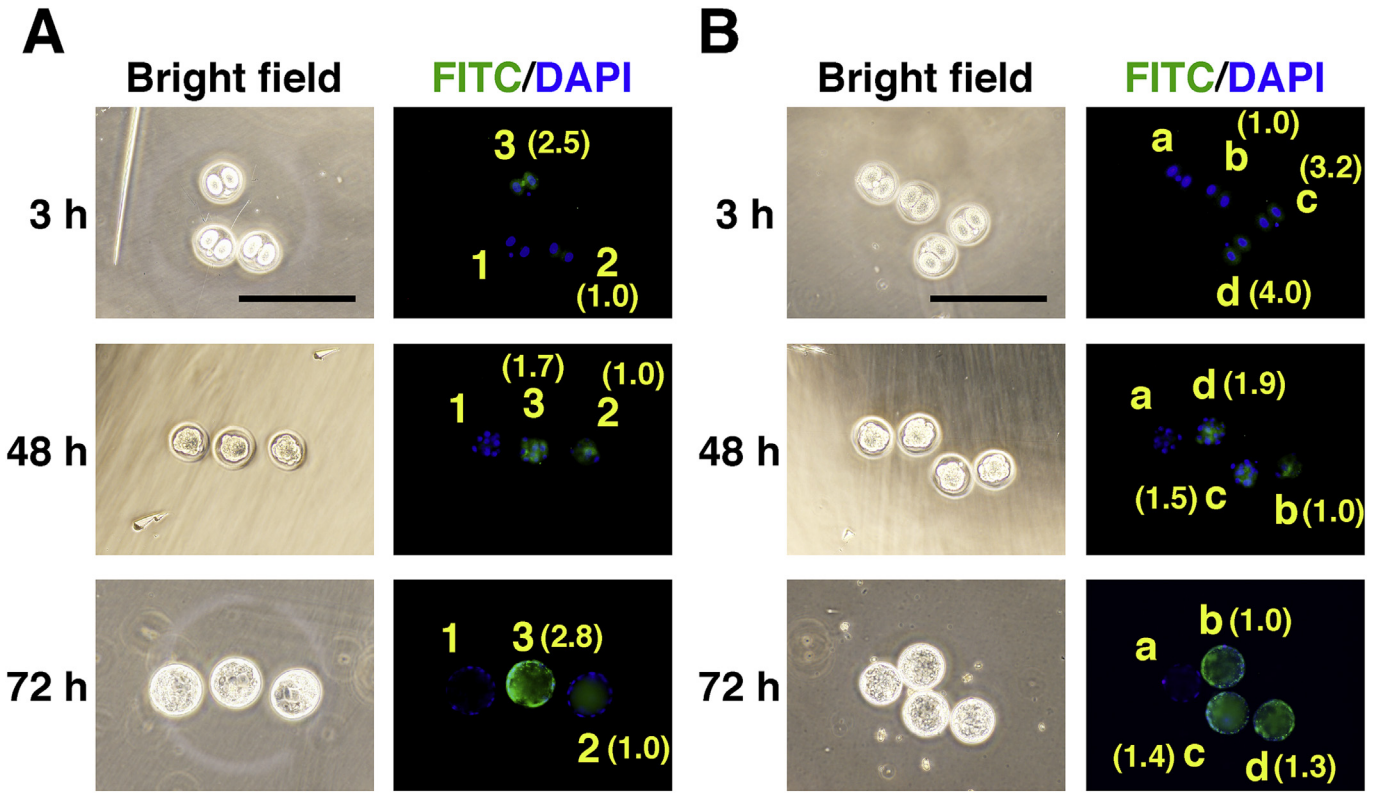

Fig. 1. Delivery of fluorescein isothiocyanate-labeled morpholino oligos by Endo-Porter into mouse 2-cell embryos cultured *in vitro*. (A) Effect of bovine serum albumin (BSA) concentrations on the incorporation of morpholino oligos (MOs) by Endo-Porter (EP). Two-cell embryos were flushed from the oviducts, and were cultured immediately with a fluorescein isothiocyanate (FITC)-labeled control MO against human thalassemic *Hbb* (10 μM) and EP (6 μM) in the presence of 3 mg or 60 μg/ml BSA for a 3-h (2-cell stage), 48-h (morula stage), or 72-h (blastocyst stage) culture period. The incorporated FITC-labeled control MO into embryos was observed after nuclear staining with 4′-6-diamino-2-phenylindole (DAPI). Cultured embryos in the respective conditions were transferred to a dish and photographed. The numbers in parentheses are relative densities of FITC signals. 1, 60 μg/ml BSA without FITC-labeled control MO (density was set as 0); 2, 3 mg/ml BSA with FITC-labeled control MO; 3, 60 μg/ml BSA with FITC-labeled control MO. (B) Effect of EP concentration on the incorporation of an FITC-labeled control MO (10 μM). a, no EP without FITC-labeled control MO (density was set as 0); b, no EP with FITC-labeled control MO; c, 2 μM EP with FITC-labeled control MO; d, 6 μM EP with FITC-labeled control MO.