APPLICATION NOTE

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DharmaFECT™ transfection reagents for siRNA transfection into embryonic stem cells

Introduction

Stem cells hold the promise of eternal propagation and unlimited differentiation into multiple cell types. Characterization of adult stem cells for many tissues such as brain, blood, skin, retina and liver has been reported. The differentiation potential of adult stem cells varies and is generally limited to the tissue of origin; this is termed multipotency. Pluripotent embryonic stem (ES) cells, on the other hand, offer the potential of differentiating into all possible cell types, making them a subject of vigorous investigation. Current research is focused on understanding the regulation of genes that are responsible for programming differentiation of these cells into specific cell types. The ability to direct differentiation is imperative for progress towards therapeutic use of ES cells in cell replacement therapy.[1]

Gene function and regulation studies in the last few years have been greatly facilitated by RNA interference (RNAi) technology. Highly functional, chemically synthesized siRNAs targeting every characterized gene in standard model animals are commercially available. For most actively dividing cells, a simple transfection of siRNA results in specific & almost complete silencing of the target gene. For siRNA delivery into non-dividing cells, physical methods such as electroporation or direct injection may be preferred over transfection. The sensitivity of ES cells to both physical and chemical methods of siRNA delivery poses a challenge to using RNAi in these cells. This Application Note describes the use of a mild and efficient lipid-based transfection reagent for siRNA delivery into mouse ES cells. The transfection procedure incorporates a modified cultivation condition for these cells to maintain their self-renewal and differentiation capacities.

Cultivation of ES cells

The cultivation condition of stem cells is critical to ensure pluripotency. Traditionally, mouse ES cells are grown on a feeder layer of mouse embryonic fibroblasts (MEFs) that are either irradiated or treated with mitomycin C to prevent proliferation. These cells provide the necessary attachment surface as well as soluble factors important for ES cell growth.[2] The presence of the feeder layer complicates mRNA or protein level analysis, as it is difficult to completely remove the feeder cells from the sample preparation. As an alternative to co-culturing on a feeder layer, many formulations of chemically defined medium for culturing ES cells are commercially available. While these are convenient to use and obviate the need for a parallel culture of MEFs, chemically defined media result in ES cells with compromised pluripotency. We have developed a culture condition for ES cells in 96-well plate format that supports pluripotency as well as facilitates siRNA transfection and gene silencing assays.

To provide optimal conditions for ES cells during transfection, we switched cells from plates with MEF feeder layers to plates that were coated with 0.1% gelatin three to four passages prior to transfection. While on MEF feeder layer, the cells were cultured in Knockout™ D-MEM and supplemented with Knockout™ Serum Replacement (see Materials table). After transfer to gelatin-coated plates, ES cells were then cultured in MEF-conditioned (medium recovered from a 24 hour culture of MEFs) to provide the growth factors normally contributed by the feeder cells. We further supplemented the conditioned medium with Leukemia Inhibitory Factor (LIF, 1000 U/mL) to promote retention of pluripotency. Under these conditions, ES-D3 cells retained their pluripotency for up to ten passages as analyzed by alkaline phosphatase (AP) assay. High AP expression is an established marker of undifferentiated stem cells.[3] Figure 1 shows that the fraction of AP-positive cells grown on gelatin-coated plates were comparable to that of cells grown on the MEF feeder layer, indicating that these cultivation conditions retain the undifferentiated state of these cells. Additionally, the following section shows that ES cells grown under these conditions are efficiently transfected with siRNA using lipid-based transfection reagents.

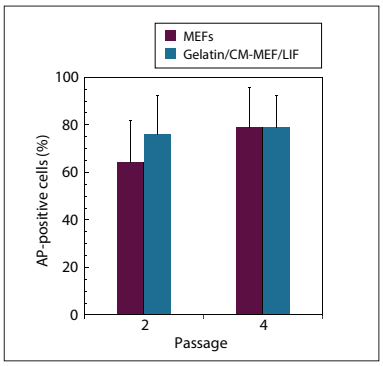

Figure 1. ES-D3 Cells Maintain Pluripotency When Cultivated without Feeder Layer. Cells were grown on either mouse embryonic fibroblasts (MEFs, red bars) or on gelatin-coated plates with MEF-conditioned medium supplemented with leukemia inhibitory factor (Gelatin/CM-MEF/LIF). Percent of alkaline phosphatase-positive cells was determined at passages two (2) and four (4) and is expressed as mean ± SD (n=3).

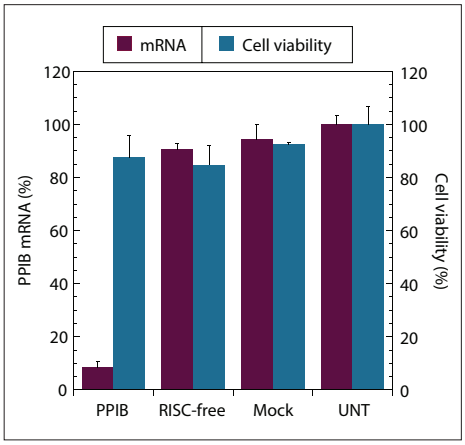

Figure 2. Efficient siRNA Transfection of ES-D3 Cells Using DharmaFECT 1. ES-D3 cells were plated at 2 × 10^3 cells/well in 96-well plates 24 hours prior to transfection using 0.2 µL/well DharmaFECT 1. Samples were either untreated (UNT) or treated with 100 nM siRNA targeting PPIB, 100 nM non-targeting siRNA (RISC-Free), or transfection reagent alone (Mock). PPIB mRNA level was measured using bDNA at 24 hours and normalized to GAPD mRNA level. Cell viability was measured by Thermo Scientific™ alamarBlue™ assay at 24 hours. All values are expressed as percent of untreated sample (mean ± SD, n = 3).

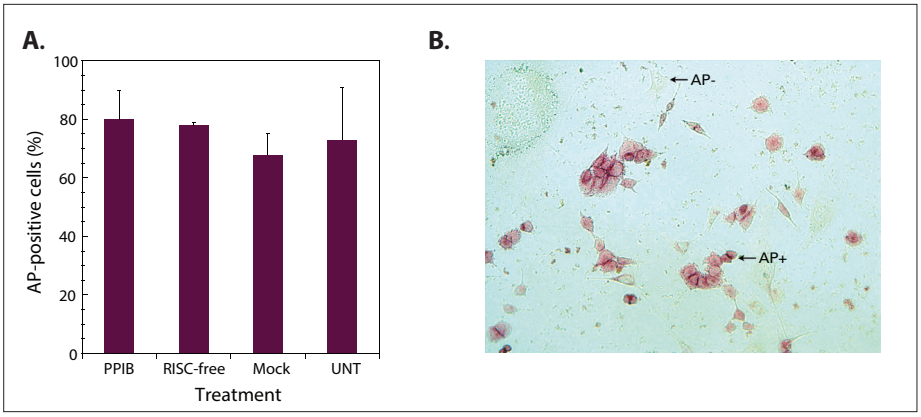

Figure 3. Pluripotency of ES-D3 Cells Transfected using DharmaFECT 1 is Maintained. ES-D3 cells were either untreated (UNT) or treated with 100 nM siRNA targeting PPIB, 100nm non-targeting siRNA (RISC-Free), or transfection reagent alone (Mock). Cells were fixed and immunoassayed for alkaline phosphatase at 24 hours using the Cell BioLabs™ StemTAG™ Alkaline Phosphatase Complete Kit (see Materials table). **A.** Quantification of alkaline phosphatase-positive cells. Percent of AP-positive cells are expressed as mean ± SD (n=3). **B.** Microscope image showing cells that are alkaline phosphatase-positive (AP+) and alkaline phosphatase-negative (AP -); magnification = 40x.

siRNA transfection into ES cells

siRNA transfection efficiency is paramount for achieving good silencing in RNAi experiments. Lipid-mediated transfection of nucleic acids is most efficient in cells that are actively dividing. Because ES cells divide slowly, this method for siRNA delivery requires careful experimentation to determine the optimal cell number to be plated, the best transfection reagent, and the optimal volume of transfection reagent. We plated 1 × 10^3, 2 × 10^3 and 4 × 10^3 cells/well in 96-well plates, and transfected 100 nM siRNA using four Dharmacon™ DharmaFECT™ siRNA Transfection Reagents at a volume range between 0.1 and 1.2 µL/well. At a density of 2 × 10^3 cells/well, all four DharmaFECT formulations provided efficient transfection at all tested volumes as measured by the silencing of the housekeeping gene PPIB (cyclophilin B, NM_011149, see Materials table).

Figure 2 shows the transfection results using 0.2 µL/well DharmaFECT 1. Over 80% silencing of PPIB was observed compared to untreated cells, indicating that high transfection efficiency was achieved while maintaining cell viability of over 80%.

With DharmaFECT volumes of 0.4 µL/well or higher, cell viability fell below 80%, which is the threshold assigned for successful, non-toxic transfection. As generally observed with other cell lines, lower cell numbers resulted in decreased cell viability; presumably due to exposure of excess transfection reagent per cell. Higher cell numbers resulted in reduced transfection efficiency due to decreased ratio of transfection reagent per cell.

In addition to maximizing cell viability of transfected ES cell, maintaining their pluripotency is also very important. An alkaline phosphatase assay was used to assess pluripotency of ES cells. Samples treated with siRNA targeting PPIB, non-targeting siRNA, or transfection reagent alone showed the same percentage of alkaline phosphatase-positive cells as untreated cells, indicating that the transfection procedure described above does not affect pluripotency of these cells (Figure 3).

Using RNAi for functional genomic analysis in ES cells

Once the transfection procedure is confirmed to not compromise the pluripotency of ES-D3 cells, RNAi can be used to identify and study genes that are involved in either maintaining pluripotency or promoting differentiation without potential false positive signals from the transfection procedure itself. We used an siRNA targeting POU5F1 (POU domain, class 5, transcription factor 1, NM_013633) to study the effect of silencing this gene on the pluripotency of ES-D3 cells. Figure 4 shows 80% reduction of POU5F1 mRNA level 48 hours after transfection of 100 nM POU5F1 siRNA, indicating efficient transfection of and silencing by the siRNA. The viability of these cells was reduced to less than 70% compared to untreated cells, suggesting that silencing of POU5F1 affected cell viability.

Increased CDX2 (caudal type homeo box 2, NM_007673) mRNA level has previously been shown to be associated with ES cell differentiation.[4] Figure 4B shows an almost five-fold increase of CDX2 mRNA level in POU5F1 siRNA-treated samples compared to untreated cells, strongly indicating that silencing of POU5F1 gene results in increased differentiation of ES cells.

Finally, the level of AP activity in these cells was also reduced about 50% compared to cells treated with non-targeting siRNA, confirming that silencing of POU5F1 reduces the pluripotency of these cells (Figure 4C). These results support previous data implicating the role of POU5F1 in maintaining pluripotency of ES cells, and demonstrates the feasibility of using chemically synthesized siRNA for studying gene function in ES cells.[5]

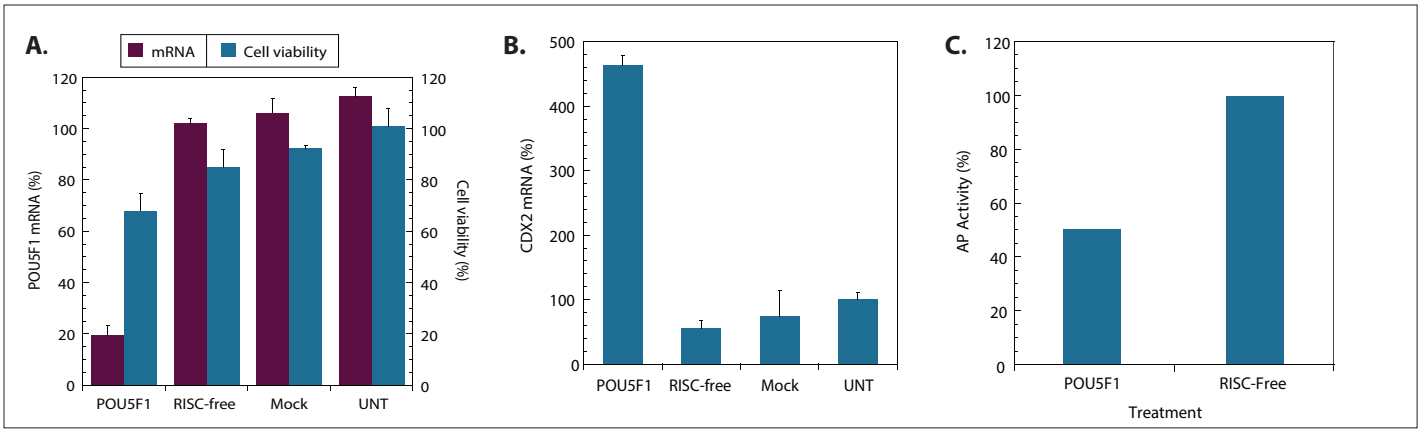

Figure 4. siRNA-mediated Silencing of POU5F1 Reduces Pluripotency of ES-D3 Cells. ES-D3 cells were either untreated (UNT) or treated with 100nM siRNA targeting POU5F1, 100 nM non-targeting siRNA (RISC-Free), or transfection reagent alone (Mock). **A.** POU5F1 mRNA level was measured using bDNA at 48 hours and normalized to GAPD mRNA level. Cell viability was measured by Thermo Scientific™ alamarBlue™ assay at 48 hours. All values are expressed as percent of untreated sample [mean +/- SD (n=3)]. **B.** CDX2 mRNA level was measured using bDNA at 72 hours and normalized to GAPD mRNA level. **C.** Cells were lysed and assayed for alkaline phosphatase level at 72 hours using the Cell Biolabs StemTAG Alkaline Phosphatase Complete Kit (see Materials table).

References

1. G. Keller, Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes & Dev.* **19**(10), 1129-55(2005).
2. F. Ulloa-Montoya, C. Verfaillie, Culture Systems for Pluripotent Stem Cells. J. *of Bioscience and Bioengineering* **100**(1),12-27 (2005).
3. J.A. Thomson, J. Itskovitz-Eldor, Embryonic Stem Cell Lines Derived from Human Blastocysts, *Science*. **282**(5391), 1145-47 (1998).
4. H. Niwa, Y. Toyooka, Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation, *Cell* **123**(5), 917-29 (2005).
5. D.C. Hay, L. Sutherland, Oct-4 Knockdown Induces Similar Patterns of Endoderm and Trophoblast Differentiation Markers in Human and Mouse Embryonic Stem Cells, *Stem Cells* **22**(2), 225-35 (2004).

Materials

Reagent	Supplier/brand	Cat #
Cell culture reagents		
ES-D3™ cells	ATCC	CRL-1934
Gelatin Solution, Type B (From Bovine Skin), 2%	Sigma-Aldrich Co.	G1393
Knockout™ D-MEM	Thermo Fisher Scientific, Inc.	10829-018
Knockout™ Serum Replacement	Thermo Fisher Scientific, Inc.	10828-028
Leukemia Inhibitory Factor (ESGRO™ [LIF])	Millipore Sigma	ESG1107
Mitomycin C	Roche Applied Science	10107409001
Mouse Embryonic Fibroblasts™	ATCC	SCRC-1008
siRNAs & transfection reagents		
DharmaFECT™ Transfection Reagents	Horizon Discovery	T-2005-01
Mouse POU5F1 (Oct3, Oct4, Otf3, Otf4, Oct-3, Oct-4, Otf-3, Otf-4, Otf3g, Oct3/4, Oct-3/4, Otf3-rs7) Dharmacon™ siGENOME™ SMARTpool™ siRNA		M-046256-00
Mouse PPIB (peptidylprolyl isomerase B, cyclophilin B) Dharmacon siGENOME™ SMARTpool™ siRNA		M-048843-00
siGENOME™ RISC-Free Control siRNA #1		D-001220-01
Detection reagents		
alamarBlue™ reagent	Thermo Fisher Scientific, Inc.	DAL1100
bDNA QuantiGene™ Reagent System Kit	Affymetrix, Inc	Target-specific
StemTAG™ Alkaline Phosphatase Complete Kit	Cell Biolabs, Inc	CBA-302

If you have any questions, contact

t +44 (0) 1223 976 000 (UK) **or** +1 800 235 9880 (USA); +1 303 604 9499 (USA)
f + 44 (0)1223 655 581
w horizondiscovery.com/contact-us **or** dharmacon.horizondiscovery.com/service-and-support
Horizon Discovery, 8100 Cambridge Research Park, Waterbeach, Cambridge, CB25 9TL, United Kingdom

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