Optimization of conditions for transfection of single stranded deoxyoligonucleotides into mouse embryonic stem cells with Metafectene and Metafectene Pro

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Introduction:

Formation of triple helix structures using chromosomal DNA has many diagnostic and therapeutic uses. One such use is for the development of a general method for gene mutation correction directly on the chromosome. A prerequisite for this approach to be a viable form of gene therapy is the successful transfection of modest sizes single stranded deoxyoligonucleotides into mammalian cells. Specifically, the use of embryonic stem cells in this endeavor holds great promise.

Unfortunately, transfection of a single stranded deoxyoligonucleotide is difficult to monitor (due to the lack of inherent marker to indicate successful transfection), and studies whereby transfection efficiencies of such a strand are measured have not been adequately performed previously. To this end, we developed a transfection assay for such deoxyoligonucleotides, and, using this assay, maximized the transfection conditions for the system in a line of mouse embryonic stem cells (E14/T).

Materials and methods:

Cells

The E14/T line of mouse embryonic stem cells was maintained without feeder cells on gelatinized plates as described elsewhere, supplemented with 15% FBS (Biofluids) and 1,000 U/mL leukemia inhibitory factor (LIF) (ESGRO; Chemicon) [1].

Transfection Assay

Transfections of single stranded deoxyoligonucleotides labeled with fluorecein isothiocyanate (FITC) were performed in duplicate for each reagent at given times before collection of all samples for evaluation by flow cytometry (so as to have live whole cells and freshly isolated nuclei for each condition). The whole cell samples were trypsinized, and the cells washed twice in PBS before resuspension in an appropriate volume of PBS for analysis. The other set of duplicate plates was used for isolation of cell nuclei using a variation of the Dignam protocol [2]. Growth media (containing the transfection reagent) was aspirated and replaced with TMSD buffer (20mM HEPES, 1.5mM $MgCl_2$, 0.25M sucrose, 1mM DTT, 0.5mM PMSF, pH 7.5). Cells were scraped from the plate and incubated at 37°C for 10 minutes, and then

placed on ice for an additional 10 minutes before nuclei were collected by centrifugation and resuspended in an appropriate volume of TMSD for analysis. All whole cell and nuclei samples were counted on a hemacytometer prior to analysis by flow cytometry. Flow cytometry on whole cells and isolated cell nuclei was performed using a FACScan flow cytometer (BD Biosciences), with excitation at 488nm. FITC fluorescence signals were collected using a 530/30 bandpass filter.

Deoxyoligonucleotides

Deoxyoligonucleotides were synthesized by the Princeton University Syn/Seq facility. Crude deoxyoligonucleotide fractions were labeled with FITC(Sigma) by covalent conjugation with a 5′-amine modified nucleotide, and purified by denaturing polyacrylamide gel electrophoresis. Labeled deoxyoligonucleotides were removed from gel slices by elution in 0.1 M sodium phosphate (pH 7.0). Final purification was accomplished by 100% acetonitrile elution from a C18 Sep-Pak reverse phase column (Waters), followed by spin evaporation. Final concentrations of labeled deoxyoligonucleotides were established using an Aviv spectrophotometer.

Transfection Reagents

Samples of Metafectene and Metafectene Pro were provided by Biontex for evaluation with the aforementioned transfection system.

Experimental procedures / transfection protocol:

4-5x10^5 E14/T mouse embryonic stem cells were seeded on 6cm gelatinized dishes in 4mL standard growth media and grown 6-24hrs at 37℃, 10% CO_2 prior to transfection. The indicated amounts of liposomal reagent (Metafectene or Metafectene Pro) and FITC-labeled deoxyoligonucleotide were each resuspended in 250µL DMEM, and then these solutions were mixed together. The mixtures were incubated at room temperature for 30 minutes before being added directly to the complete growth medium of the E14/T cells. At the indicated times after transfection, cells and/or nuclei were prepared for flow cytometry as described above.

Results and discussion:

Maintenance of an undifferentiated cell state

When working with embryonic stem cells, an undifferentiated state must be maintained at all times. Neither transfection with Metafectene nor Metafectene Pro inherently altered this state. Visual inspection of the cells prior to harvesting for flow cytometry (data not shown) indicates that the general morphology of the cells is not altered by transfection. Additionally, the proliferative capacity of the cells is not greatly altered. While cell counts indicate that slightly fewer cells generally are present in transfected samples (Fig. 1), less than a 1-fold change is not deemed a significant decrease. The difference in cell number is most likely due to a growth lag, as inspection of the flow cytometry profiles (data not shown) does not indicate an excess of dead cells compared to untransfected controls.

Since serum starvation results in differentiation of embryonic stem cells, the media contained the standard 15% FBS. The presence of serum does not appear to drastically inhibit transfection in differentiated cells (M. Ulasli, personal observation), and it does it seem to be a detriment here either.

Ratio of liposomal reagent:growth media volume

Two parameters were maximized in this study: the ratio of liposome to volume of growth medium (liposomal saturation of the medium) and the ratio of deoxyoligonucleotide to liposome (packaging capacity of the liposomal reagent).

Using conditions for differentiated cells previously established in our laboratory, a preliminary time trial for the transfection of E14/T mouse embryonic stem cells was performed (data not shown). This trial indicated that a transfection time of 18hrs would be sufficient to see substantial transfection at less than maximal conditions.

In order to optimize the liposomal saturation of the cell medium, the volume of cell growth medium is held constant at 4mL, and a fixed ssDNA:liposome ratio of 0.1µg oligo:1µL reagent was used while increasing the amount of liposomal reagent (Metafectene or Metafectene Pro) added. Thus, as can be seen in Figure 2, the optimal ratio of liposome:medium is 1-2µL liposomal reagent per 1mL growth medium for both reagents tested.

Ratio of ssDNA:liposomal reagent

While the previous study indicated that 1-2µL liposome/mL growth medium is the optimal saturation level, the saturation level of the liposomal reagent was reduced to 0.5µL reagent/mL medium due to limited amounts of deoxyoligonucleotide available, and inability of preliminary trials (data not shown) to reach a saturating amount of ssDNA with the larger liposomal reagent:growth medium ratio. Figure 3 shows that 1-2µg of ssDNA per 1µL liposomal reagent (Metafectene or Metafectene Pro) is the optimal ratio of ssDNA:liposomal reagent for transfection with both reagents tested. Note that for the manufacturer recommended range of 1µg nucleic acid:2-7µL liposome (Metafectene or Metafectene Pro) results in the *least* transfection according to this study.

Time course study of transfection

In this time trial, the conditions of 0.2µg ssDNA:1µL transfection reagent:1mL growth medium were used (due again to the limiting amount of deoxyoligonucleotide). Figure 4A shows that transfected nuclei are readily detectable for at least 48hrs after transfection. When these raw data are combined with the nuclei counts performed prior to analysis, the persistence of the deoxyoligonucleotide in the cells is seen (Figure 4B). Thus, transfection seems to be achieved within the first 24hrs after addition of the liposomal reagent, and the ssDNA persists in the nucleus for an additional 24hrs.

Conclusion / summary:

The data appear to show that transfection of our single stranded deoxyoligonucleotide is marginally better with Metafectene Pro than with Metafectene over time. Moreover, transfection appears to occur more rapidly with Metafectene Pro as opposed to with Metafectene. Persistence of the deoxyoligonucleotide is identical for both reagents.

The optimization trials themselves also indicate that a given volume of Metafectene Pro saturates the growth medium less than the same volume of Metafectene. Additionally, the packaging capacity of a given volume of Metafectene Pro is lower than that of Metafectene.

References:

1. Niwa, H., Burdon, T., Chambers, I., Smith, A. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**: 2048-2060.

2. Dignam, J.D., Lebovitz, R.M., Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nuc. Acids Res.* **11**: 1475-1489.

Appendix: Tables and/or figures:

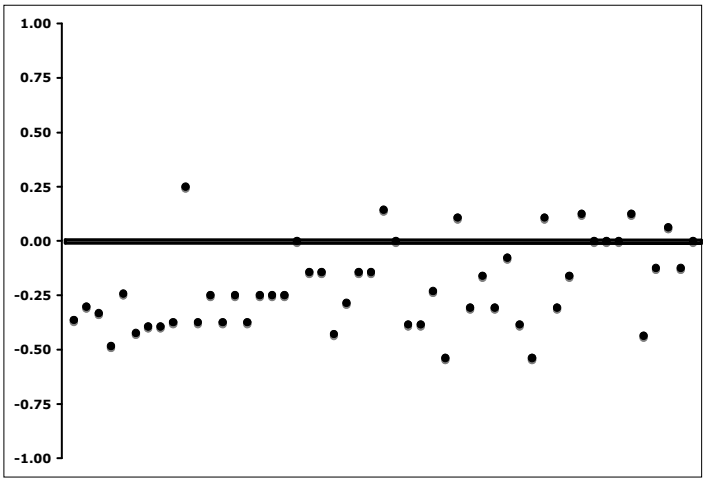


Figure 1: Fold change in number of cells assayed for transfection. Fold change of transfected cell sample is relative to untransfected control for a given experiment. Graph displays data from both transfection reagents and for all conditions described in subsequent figures.

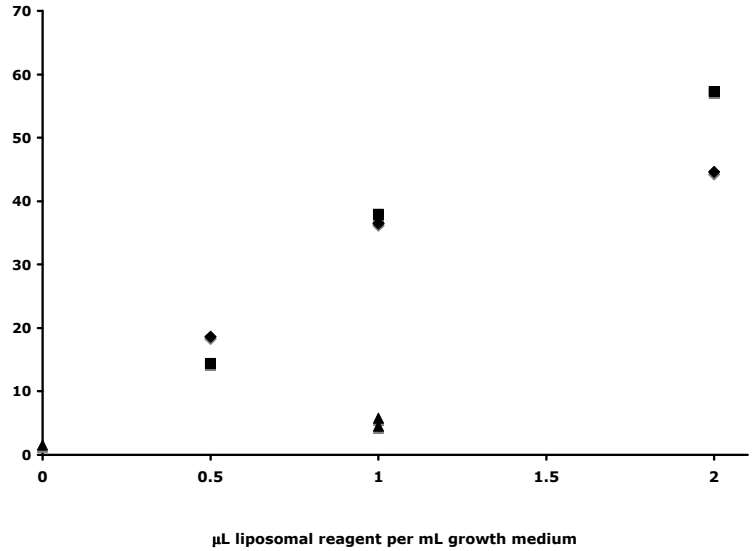


Figure 2: Exploration of liposome:media ratio. Cells were transfected using a fixed ratio of 0.1µg ssDNA:1µL tranfection reagent. After 18hrs, cells were trypsinized and examined for FITC fluorescence by flow cytometry. Only whole cells were examined in this trial, as the nuclei isolation protocol had not been sufficiently perfected at this point. Diamonds: Metafectene, Squares: Metafectene Pro, Triangles: mock transfections and untransfected samples.

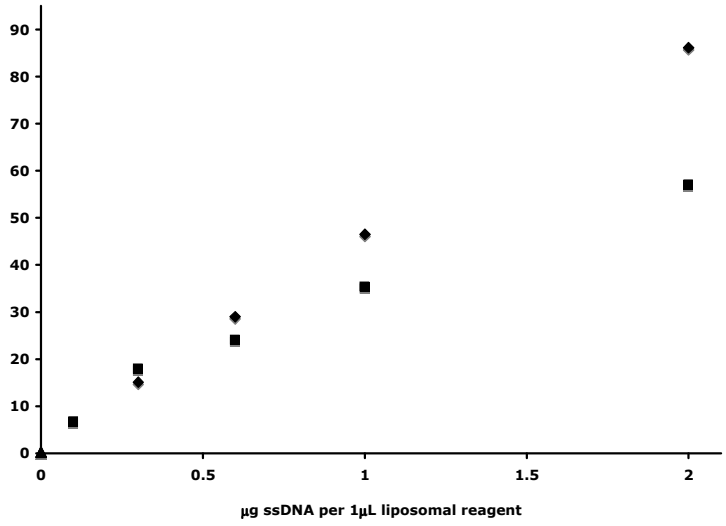
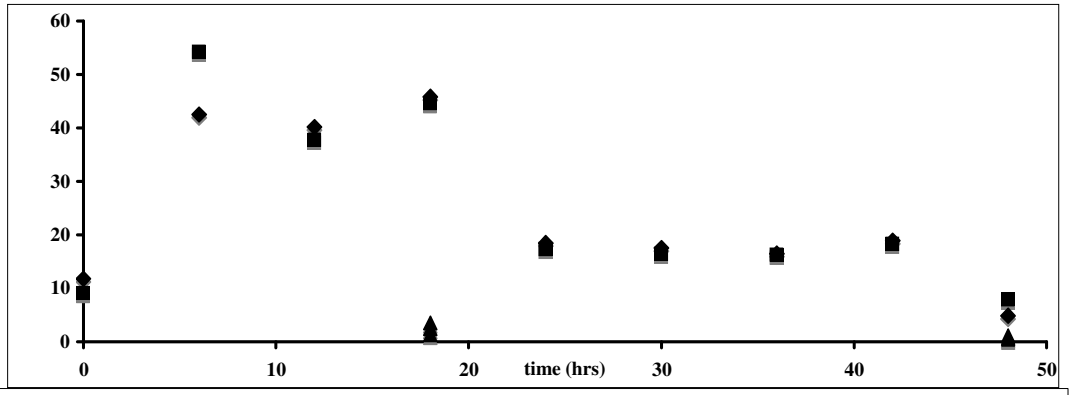


Figure 3: Exploration of DNA:liposome ratio. Cells were transfected using a fixed ratio of 0.5µL transfection reagent:1mL growth medium. After 18hrs, nuclei were isolated and examined for FITC fluorescence by flow cytometry. Diamonds: Metafectene, Squares: Metafectene Pro, Triangles: mock transfections and untransfected samples.

A.



B.

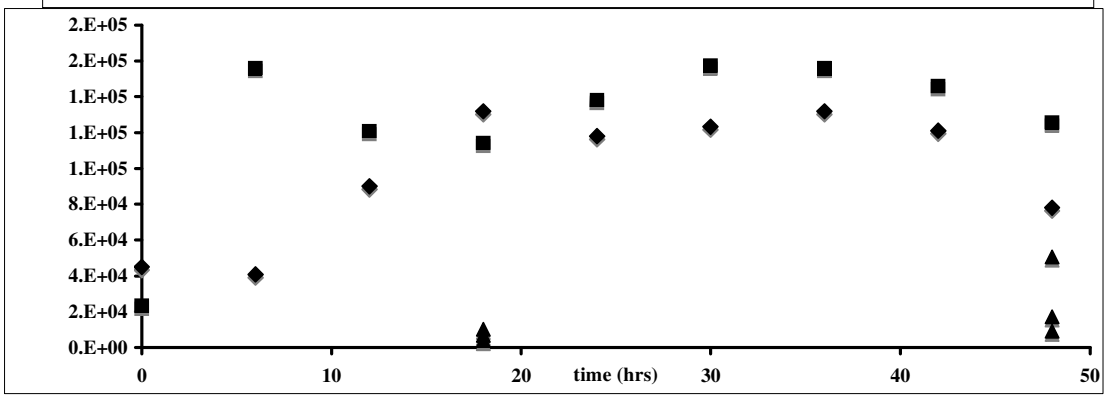


Figure 4: Transfection timecourse. Cells were transfected using a ratio of 0.2µg ssDNA:1µL reagent:1mL growth medium. After the given amounts of time, nuclei were isolated and examined for FITC fluorescence by flow cytometry. (A) Percent of FITC-positive nuclei over time. (B) Number of FITC-positive nuclei over time. Diamonds: Metafectene, Squares: Metafectene Pro, Triangles: mock transfections and untransfected samples.

Table 1: Comparison of optimal packaging ratios of different nucleic acids with Metafectene and Metafectene Pro.

Nucleic Acid	µg/µL reagent*	pmol/µL reagent
dsDNA	0.2	0.05
ssRNA	0.2	15
ssDNA	1	100

*: Ratios were approximated using data from the Metafectene and Metafectene Pro Guidebooks supplied by Biontex, as well as data presented here.

†: Calculations assumed 5kb dsDNA plasmid, 40nt ssRNA, and the 31nt ssDNA+FITC used in this study. Values are approximations intended for scale comparisons only.