Polymeric Transfection Reagents for shRNA delivery *in vitro* and *in vivo*

1Gwen D. Fewell, 2Majed Matar, 2Jason G Fewell, 1Troy Moore and 1Rusla Du Breuil
1Open Biosystems, Inc Huntsville, AL and ***2****Expression Genetics, Inc., Huntsville, AL*

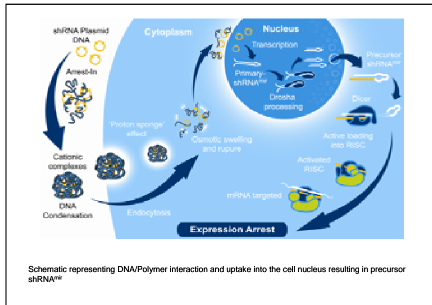
INTRODUCTION

Gene silencing using RNAi is critically dependent on highly efficient delivery of silencing triggers into cells. Certain PEI-based polymeric delivery systems can protect DNA in the cytoplasm and promote efficient entry into nuclei of transfected cells. Arrest-In™ transfection reagent is a proprietary lipo-polymeric formulation, optimized for transfection of cultured eukaryotic cells with shRNA plasmid DNA. The lipid component of Arrest-In allows greater uptake efficiency into cells through enhanced cell membrane interaction. Once in the cells, Arrest-In promotes endosomal escape and entry of the shRNA containing plasmid into the nucleus where it is transcribed and enters the cytoplasm to be processed by the endogenous RNAi machinery into functional siRNAs (FIG 1).

Arrest-In produces high transfection efficiencies with extremely low cytotoxicity. *In vitro* data will be presented showing transfection efficiencies in a variety of cell lines including a comparison with other popular reagents.

A modified Arrest-In is under development that is designed specifically for *in vivo* RNAi studies. This reagent produces highly condensed particles that protect transfected DNA from endogenous DNAase activity producing a longer circulating half-life. *In vivo* data in mice will be presented showing robust knockdown of co-transfected reporter protein in lung, liver and spleen after intravenous injection of shRNA complexed with *in vivo* Arrest-In.

Figure 1.



Schematic representing DNA/Polymer interaction and uptake into the cell nucleus resulting in precursor shRNAmir

METHODS

***In Vitro* Gene Transfer:** Transfection activity of the polymeric delivery systems were examined in various cell lines. In general, cell cultures were prepared to 80% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Transfection complexes containing 1-2 μg plasmid and the polymer delivery system were formulated at an N:P ratio of 20:1 which corresponds to an approximate 5.0:1 (Polymer:DNA) ratio on a wt:wt basis. Transfection complexes were added into each well in the absence of 10% FBS for 6 hours in a CO_2 incubator. Transfection medium was subsequently replaced and cells were incubated for up to 40 hours prior to analysis. The manufacturers instructions were followed when using Lipofectamine 2000® (Invitrogen, Carlsbad, CA), Exgen500® (Fermentas, Hanover, MD) or FuGene® (Roche Diagnostics, Basel, Switzerland).

Cytotoxicity and Protein Determinations: The cytotoxicity of gene transfection was assessed using a cell proliferation assay (Promega Corporation, Madison, WI). Cells were transfected under the same conditions as previously described. To perform the assay, 20μl of CellTiter 96® reagent was added to each well, plates were incubated for 4 hours at room temperature and read at 490 nm on an ELISA plate reader. The relative percent cell viability was calculated as described in the manufacturers protocol. Luciferase activity determinations were from cell lysates using a commercially available assay (Promega Corporation, Madison, WI.). Activity was measured using a micro plate luminometer (Berthold Detection systems USA, Oak Ridge, TN). Mouse VEGF protein levels were determined by ELISA using a commercially available ELISA assay from R&D systems (Minneapolis, MN).

***In Vivo* delivery:** The transfection complexes containing luciferase plasmid, luciferase shRNA plasmid and non-silencing shRNA plasmid were administered into the tail vein of normal ICR mice (Harlan, Houston, TX). The total plasmid concentration in the formulation was fixed at 0.1 mg/ml to yield a 30 μg dose in 0.3 ml injection volume. Animals were euthanized after 24 hours and the lungs, livers and spleens were harvested. Tissues were homogenized in 1 ml TENT buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, 0.5% Triton X-100), and assayed for VEGF protein levels. All animal experiments conformed to accepted practice outlined by the Institute of Laboratory Animals Resources and followed an accepted IACUC protocol.

RESULTS

➢Arrest-In and Fluorescent Arrest-In were synthesized from low molecular weight linear polyethyleneimines (LPEI) that were generated by a uniform synthesis scheme in order to minimize molecular and structural heterogeneity. The low molecular weight LPEI were then cross linked via biodegradable linkages to produce a multi-block copolymer. Addition of an auxiliary lipid ligand completed the synthesis process. Arrest-In produces highly efficient plasmid DNA delivery in multiple cell lines (FIG 2.). Transfection efficiency compares very favorably with other commercially available transfection reagents.

➢Use of rhodamine labeled fluorescent Arrest-In allows for visualization of transfection events in real time in addition to promoting high transfection rates (FIG 2.).

➢Minimal cytotoxicity is associated with use of either Arrest-In and Fluorescent Arrest-In (FIG 3A.) and Arrest-In transfection reagents are equally efficient in the presence of serum (FIG 3B).

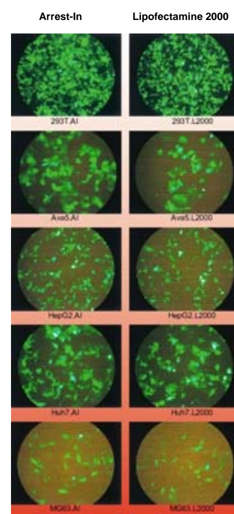
➢In vitro in co-transfection studies in COS-1 cells using Arrest-In led to >90% knockdown of therapeutically important VEGF protein following transfection of VEGF shRNA plasmid (FIG 4A). Stable human β -secretase (BACE) knock-down cells (neuroblastoma) were produced using Arrest-In transfection with 5 different shRNAmir plasmid constructs (FIG 4B).

➢A new polymer delivery system that is distinct from Arrest-In is currently being evaluated for in vivo use. Initial results indicate that intravenous (IV) delivery using this polymer can safely produce reporter gene knockdown rates of 75%-94% in multiple organs (FIG 5). Future experiments will be performed to continue to evaluate the full potential of the novel polymeric delivery system for in vivo RNAi applications.

Figure 2.

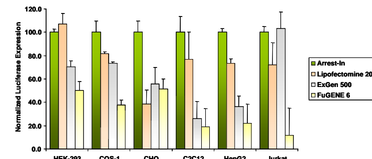
High transfection efficiency in multiple cell lines

Arrest-In **Lipofectamine 2000**



Cell lines were transfected with plasmid encoding for green fluorescent protein (GFP) using recommended protocols. GFP expression was visualized using fluorescent microscopy. The cell lines used were: human embryonic kidney (293T), HCV subgenomic-replicon cells (Ava5), human hepatocarcinoma (HepG2), human hepatoma cells (Huh7) and human osteoblastic cell (MG63).

Comparison to commercially avialable reagents

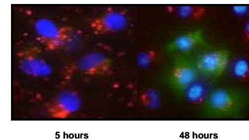


Various cell lines were transfected with a plasmid encoding for luciferase gene using commercially available transfection reagents and compared to Arrest-In. Luciferase expression levels (±SD) were normalized to those achieved using Arrest-In.

Cell lines successfully transfected with Arrest-In

Hek 293	Hek293T	Cos	Jurkat	Hela
CHO	HepG2	Mouse ES	Huh7	5ySy
PC12	MO313	Ava5	MG63	3T3
MCF7	HUVEC	RAW	PC12	NT2
LNCaP				

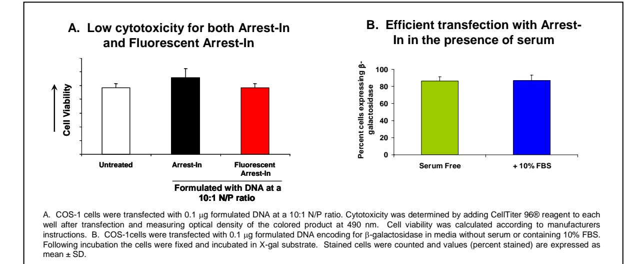
Fluorescent Arrest-In: Allows for visualization of transfection events in real time and promotes efficient gene delivery.



5 hours **48 hours**

Cos-1 cells transfected with a plasmid encoding for green fluorescent protein (GFP) complexed with fluorescent Arrest-In. Cells were visualized after incubation times of 5 hours and 48 hours. Cells were labeled with DAPI to visualize nuclei (blue).

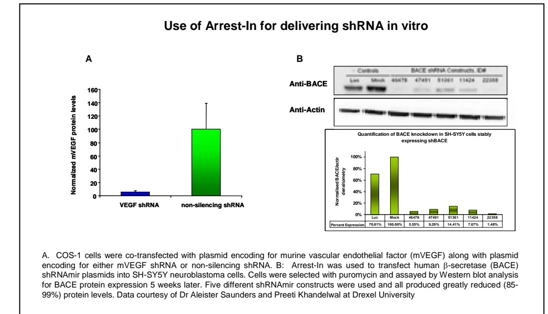
Figure 3.



A. COS-1 cells were transfected with 0.1 μg formulated DNA at a 10:1 N/P ratio. Cytotoxicity was determined by adding CellTiter 96® reagent to each well after transfection and measuring optical density of the colored product at 490 nm. Cell viability was calculated according to manufacturers instructions. B. COS-1cells were transfected with 0.1 μg formulated DNA encoding for β-galactosidase in media without serum or containing 10% FBS. Following incubation the cells were fixed and incubated in X-gal substrate. Stained cells were counted and values (percent stained) are expressed as mean ± SD.

Figure 4.

Use of Arrest-In for delivering shRNA in vitro

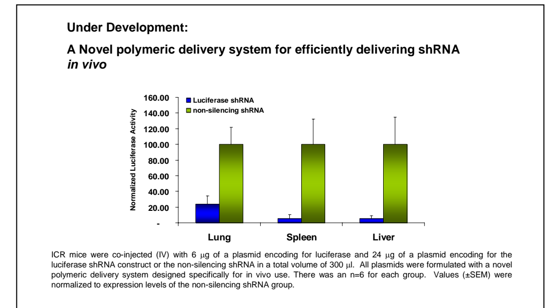


A. COS-1 cells were co-transfected with plasmid encoding for murine vascular endothelial factor (mVEGF) along with plasmid encoding for either mVEGF shRNA or non-silencing shRNA. B: Arrest-In was used to transfect human β-secretase (BACE) shRNAmir plasmids into SH-SY5Y neuroblastoma cells. Cells were selected with puromycin and assayed by Western blot analysis for BACE protein expression 5 weeks later. Five different shRNAmir constructs were used and all produced greatly reduced (85-99%) protein levels. Data courtesy of Dr Aleister Saunders and Preeti Khandelwal at Drexel University

Figure 5.

Under Development:

A Novel polymeric delivery system for efficiently delivering shRNA *in vivo*



ICR mice were co-injected (IV) with 6 μg of a plasmid encoding for luciferase and 24 μg of a plasmid encoding for the luciferase shRNA construct or the non-silencing shRNA in a total volume of 300 μl. All plasmids were formulated with a novel polymeric delivery system designed specifically for in vivo use. There was an n=6 for each group. Values (±SEM) were normalized to expression levels of the non-silencing shRNA group.