Lipofectamine Mediated Gene Delivery: Cationic Lipid Helps to Overcome the Cell-Surface Barriers in Hepatocytes

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Abstract

The objective of this study is to find effective siRNA delivery vehicle targeting liver, specifically into hepatocytes. Recently developed cationic lipid lipofectamine provide a very high density of positive charges along its backbone and have been reported to be effective in siRNA delivery to target liver cells. We compared lipofectamine mediated siRNA delivery with a cationic polymer based approach (jetPEI), in cultured fresh rat hepatocytes and human embryonic kidney cell line HEK293. We tested plasmids encoding either GFP or luciferase, and a TEX615 red dye labeled oligo to examine efficient translocation into primary hepatocytes in culture. Our results clearly demonstrated the higher efficacy of lipofectamine over jetPEI in targeting hepatocytes for both plasmid and labeled oligo delivery.

1. Introduction

Lipid based nanoparticle or cationic lipid is being extensively used to deliver siRNA or plasmid DNA in targeted tissues/cells. In this system, effective transfer happens through endocytosis pathways that include proton sponge effect and osmotic imbalance. RNA interference (RNAi) is one among most recently use gene-silencing mechanism elicited by microRNA and small interfering RNA (siRNA) that is initiated by the enzyme Dicer which cleave double stranded RNA molecule (dsRNA) (Fire et al., 1998). The induction of RNA induced silencing complex (RISC), a endonuclease, cleaves the complementary mRNA corresponding to siRNA/ or microRNA binding sites. All cationic lipids form multi-lamellar structure with negatively charge siRNA stands, generating multi-lamellar lipid-DNA complexes (lipoplexes) with positively charged lipid bilayers separated from one another by sheets of negatively charged siRNA strands (Schroeder et al., 2010).

It has been found that different nanoparticles have vastly different chemical structures and physical properties and that a majority of the DNA-carrier complexes is taken up into the cells by endocytosis including polyethylenimine (Felgner et al.,

1994, Godbey et al., 1999). During endocytosis most of the lipid DNA aggregates deposit in the vesicular compartment. Transfer from cytoplasm to nucleus is a key regulated process currently limiting gene therapy. Success of gene therapy depends on escape of lipid-DNA degradation from endosome and contributes to transfection efficiency (Simoes et al., 1999, Zabner et al., 1995).

It has been suggested that non-lamellar phase of lipoplexes, intracellular lipid mediated dissociation of siRNA and protect from endosomal membrane is key process of translocation from cytoplasm to nucleus (Zabner et al., 1995). Nanoparticle research is focused on developing carriers for DNA delivery has been to facilitate the endosomal escape of the DNA-carrier complexes prior to their degradation in the lysosomes (Hoekstra et al., 2007, Zhou et al., 2007). In this method of cationic lipid-based DNA delivery, the lipids function by compacting plasmid DNA into DNA-containing liposomes or lipoplexes. The lipoplex particles facilitate cellular uptake of DNA primarily via endocytosis following binding of positively charged lipid-DNA lipoplexes to the negatively charged cellular surfaces. As the majority of DNA lipoplexes degrade in the endolysosomes, most of the efforts to enhance DNA

transfection have been focused on stimulating the escape of DNA lipoplexes from the endolysosomes (Lu et al., 2009). Primary hepatocyte culture is very difficult system to transfect DNA or RNA. Recently, smaller nanoparticles of sizes less than 100nm and modified with hydrophilic materials have been found to be effective in reducing Kupffer cell uptake (Schroeder et al., 2010). Efficient exogenous gene expression was achieved using a recently developed cationic liposome, TFL-3, and was compared with commercially developed Lipofectamine (Invitrogen) in primary cultured rat hepatocyes as a model of non- or less frequently dividing mammalian cells (Nguyen et al., 2003, 2005). A key finding is that the degree and rate of gene expression was dependent on the time of culture prior to lipofection, post-lipofection, and on the density of cells in a dish. Furthermore, it was shown that the extent of gene expression was not necessarily related to the amount of plasmid DNA delivered to nuclei. Successful lipofections were observed for all plasmid DNA tested, and the efficiencies were superior to that of commercially available Lipofectamine. These results indicate that for achieving sufficient gene expression in rat hepatocytes the culture time prior to and following lipofection, which is related to the biological condition of the cells, may be one major factor affecting efficient gene expression in non-dividing cells. Gene expression in mouse hepatocytes by TFL-3 was successful and the level was higher than those in rat hepatocytes (Nguyen et al., 2005).

Here, we compare the transfection efficacy of lipofectamine to a polyethylenimine based cationic vehicle (jetPEI). Lipofectamine™ 2000 (Invitrogen) cationic lipid based transfection reagent is a proprietary formulation for the transfection of nucleic acids (DNA and RNA) into eukaryotic cells and is reported for highest transfection efficiency in many cell types and formats. JetPEI-Hepatocyte™ (Polyplus Transfection Inc.) is a polyethylenimine base cationic polymer formulation has recently developed for *in vitro* delivery of plasmid and siRNAs specifically in primary hepatocyte culture. We tested the delivery of siRNA, pMAX-GFPand PGL3 luciferase plasmids into primary rat hepatocytes during early time phase and compared to HEK293 cell lines with short interfering RNAs (siRNA) for use in RNA interference (RNAi) studies.

2. Materials and Methods

2.1. Animals

Present experiments were carried out in male Sprague Dawley rats purchased from Harlan (Indianapolis, IN). Animals were acclimatized for at least 1 week following their arrival in our facility. The body weights of the animals used ranged from 275-325 g. All animals were maintained on lab-chow until used. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University, Philadelphia, which is an AAALAC-accredited facility.

2.2. Preparation of hepatocytes

Hepatocytes were collected as per protocol for the collection of kupffer cells were prepared as previously described by Ponnappa et al 1998, which is a modification of the procedure originally described by Bautista et al., 1992.

2.3. pMAX-GFP plasmid transfection

One day primary cultures of hepatocyte, maintained in Williams' Medium E supplemented with 10% fetal bovine serum (FBS), 30mM pyruvate and antibiotics, were exposed to plasmid- encoded green fluorescent protein (GFP) pMAX-GFP(1 μg per well in a 24 well plate). One day cultures of HEK293 cells, maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 30mM pyruvate and antibiotics, were exposed to plasmid- encoded green fluorescent protein (GFP) pMAX-GFP (1 μg per well in a 24 well plate). Prior to incubation, the GFP-plasmid (pMAX-GFP) was polymerized with jetPEI-Hepatocyte™ (Polyplus Transfection Inc) as per standard protocol. The cells were exposed to the plasmid-JetPEI-Hepatocyte™ preparation for 24, 48 and 72h, at the end of which, the cells were fixed in 2% paraformaldehyde and the intensity of the GFP was recorded using a Olympus Fluorescent Microscope.

2.4. PGL3 plasmid transfection

One day primary cultures of hepatocytes were exposed to PGL3 plasmid-encoded luciferase activity (0.5 to 8 mg per well in a 24 well plate). One day cultures of HEK293, were exposed to PGL3 plasmid-encoded luciferase activity (4 microgram per well in a 24 well plate). Prior to incubation, the PGL3 plasmid was polymerized with Lipofectamine as per standard protocol. The cells were exposed to the plasmid-Lipofectamine preparation for 72h, at the end of which, they were harvested and washed with PBS followed by determination of luciferase activity by Steady-Glo® Luciferase Assay System (Promega) using standard protocol.

2.5. Labeled siRNA oligo transfection

One day primary cultures of hepatocyte and HEK293 cells were exposed to 20nM final concentration of TEX615 labeled siRNA oligo in a 24 well plate. Prior to incubation, the TEX615 labeled siRNA oligo was polymerized with Lipofectamine as per standard protocol. Total 1×10^6 cells were exposed to the plasmid-Lipofectamine preparation for 6, 12, 24 and 48 hour, at the end of which, they were fixed in 2% paraformaldehyde and the intensity of the TEX615 was recorded using a Olympus

Fluorescent Microscope.

2.6. Reagents

Lipofectamine™ 2000 Transfection Reagent is designed to target liver cell. (Catalog Number: 11668-019).TEX615 dye labeled siRNA was designed to evaluate red dye contrast and localization in side cells. A maxGFP vector is a newly discovered green fluorescent protein (GFP) derived from the copepod Pontellina sp. maxGFP, just like eGFP, can be used as a positive control to monitor transfection efficiency in cells of interest. Cells transfected with amaxa's new vector pMAX-GFPrevealed comparable transfection efficiencies and mortalities. JetPEI-Hepatocyte™ was obtained from Polyplus-transfection Inc. (Catalog Number: 102-01N).

3. Results and Discussion

We first tested the efficacy of JetPEI-heypatocyte in transfecting a GFP coding plasmid into hepatocytes. Zanta et al. (1997) first developed and used this polyethylenimine (PEI) galactose conjugated cationic polymer that transfects hepatocytes by a distinct mechanism of asialoglycoprotein receptor mediated endocytosis, and is different from lipofectamine mediated transfection. We found a time-dependent increase in fluorescence intensity of pMAX-GFP plasmid from 24 h to 72 h, indicative of efficient translocation of the plasmid into hepatocytes (Plate 1). Primary hepatocytes are mostly non-dividing cells in regular growth medium and difficult to transfect but our present experiments show transfection of GFP plasmid as early as 24 hours. We compared this efficacy to transfecting the same plasmid using lipofectamine into HEK293 human embryonic kidney cells. As shown in Plate 2., there was a time-dependent increase in fluorescence intensity measured at 24h, 48h, and 72h, indicative of efficient translocation of the plasmid into HEK293 using Lipofectamine. Recently lipofectamine has been reported to use in siRNA mediated knockdown of mu-calpain in Fanconi anemia (FA-A) at similar time scale (McMahon et al., 2009). We quantified the fluoresce intensities using ImageJ software developed by NIH. Comparison of hepatocytes and HEK293 cell lines clearly demonstrate HEK293 cell lines transfected more efficiently by 24 hour. However, hepatocytes were transfected at a lower level by 24 hour with continuous increase in signal intensity until 72 hours (Figure 1).

We then tested transfection of PGL3-Lipofectamine cationic particles into hepatocytes in a 72-hour culture. We carried out experiments to optimize the dose dependency and time course for delivery and assessed the selectivity of the delivery to parenchymal cells. Levels of luciferase activity in the primary hepatocyte culture was followed over several days after treatment. There was a similar luciferase activity for 0.5 to 8 microgram of PGL3 plasmid, indicative of efficient translocation of the plasmid into hepatocytes using Lipofectamine (Figure 2a). As
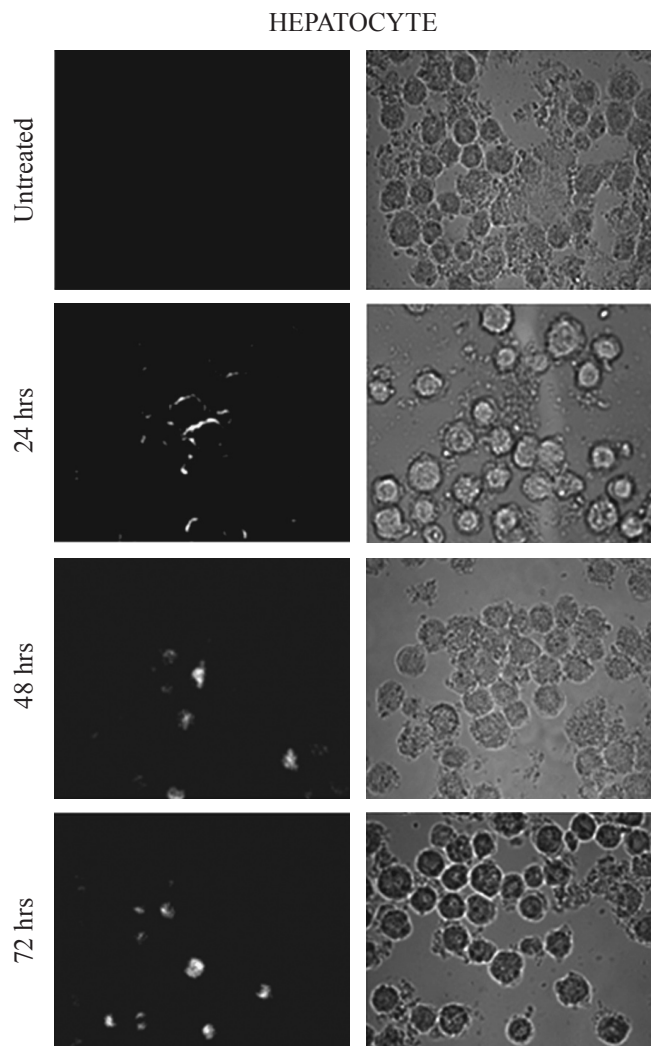
HEPATOCYTE

Plate 1: jetPEI-Hepatocyte mediated delivery and expression of pMAX-GFPplasmid in primary hepatocyte culture

shown in the Figure 2b, there was a highly significant luciferase activity for 4 microgram of PGL3 plasmid, indicative of efficient translocation of the plasmid into HEK293 cell lines as compared to that of primary hepatocyte cultures. We conclude that HEK293 cell line is easy to transfect with both pMAX-GFPand PGL3 vector and yields higher expressions of these marker proteins as compared to primary hepatocytes.

We tested delivery of labeled siRNA using lipofectamine. As shown in the Plate 3a there was a time-dependent increase in fluorescence intensity, indicative of efficient translocation of the TEX615 label siRNA into hepatocyes using Lipofectamine. By 6 hours primary hepatocyte cultures become transfected with this dye and it is optimum at 12 hours after which there is no further increase in the transfection. Plate 3b shows a time-dependent increase in fluorescence intensity TEX615 label siRNA using lipofectamine, indicative of efficient translocation of the plasmid into HEK293. As compared to primary
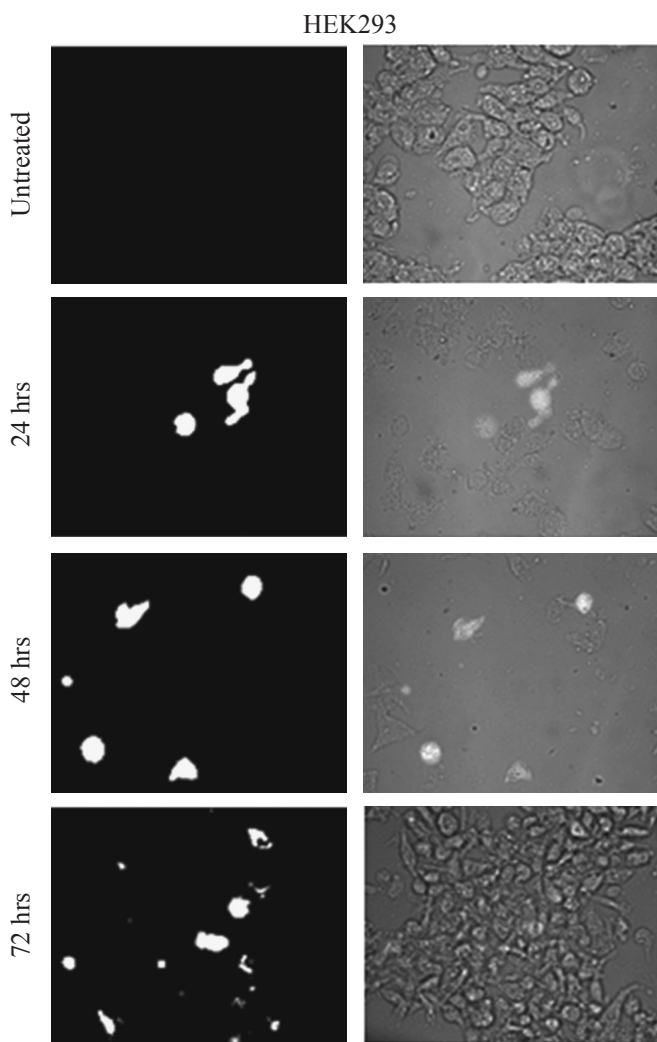
HEK293

Plate 2: Lipofectamine mediated delivery and expression of pMAX-GFPplasmid in HEK293 cell lines

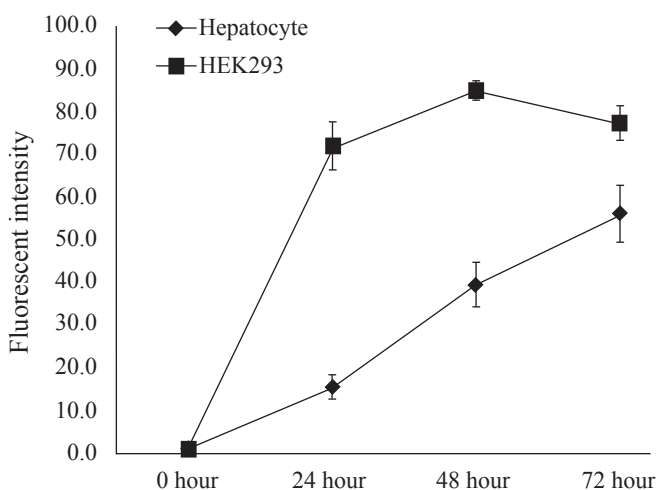
Figure 1: Quantification of fluorescent intensity of pMAX-GFPin hepatocyte and HEK293 cell lines
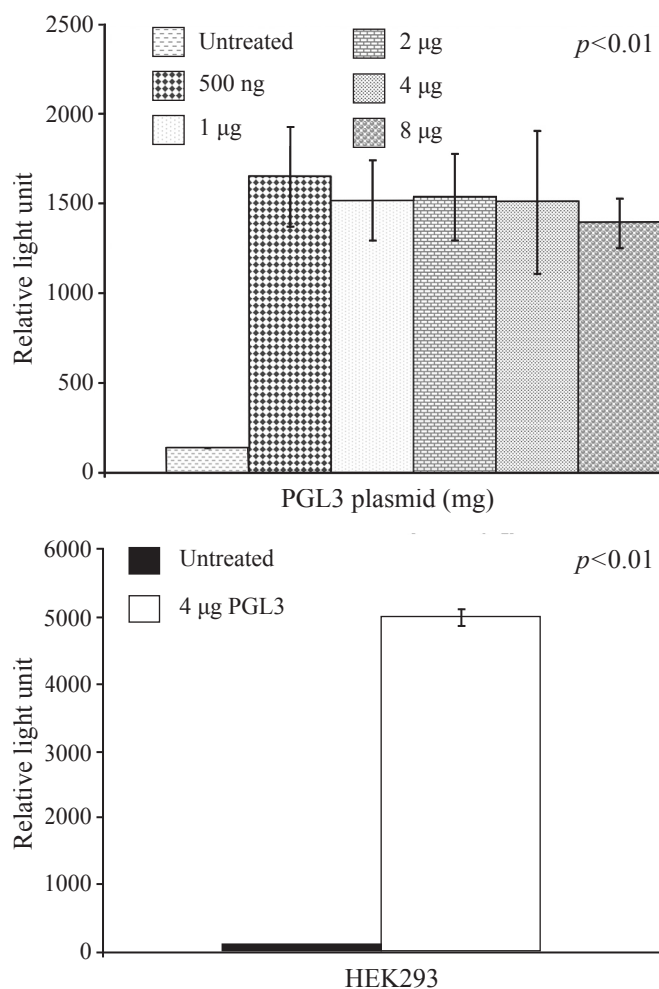

Figure 2: (a) Delivery of Luciferase plasmid PGL3 into rat Hepatocyte by Lipofectamine. (b) Delivery of Luciferase plasmid PGL3 into HEK293 cell by Lipofectamine

hepatocyte, HEK293 cell lines become transfected at 6 hour very efficiently and continue to show strong signal for rest of the time points. We quantified the fluorescent intensity of TEX615 dye using ImageJ software developed by NIH. Comparison of primary hepatocytes and HEK293 cell lines clearly demonstrates that HEK293 cell lines transfected very early at 6 hour post transfection and it is almost similar till 24 hour and decrease at 48 hour (Figure 3). In contrast, hepatocytes transfected at 6 hour but reach optimal levels at 12 hour and remains the same until 48 hours post transfection.

Lipofectamine has been reported for successful siRNA delivery in targeted cells by several studies. For example, one study reported Lipofectamine mediated knockdown of μ-calpain in Fanconi anemia (FA-A) cells by siRNA to restore alphaII spectrin levels and correct chromosomal instability and defective DNA interstrand cross-link repair (Zhang et al., 2010). FA-A and normal cells were transfected with μ-calpain siRNA or non-target siRNA using Lipofectamine 2000 Transfection Re-
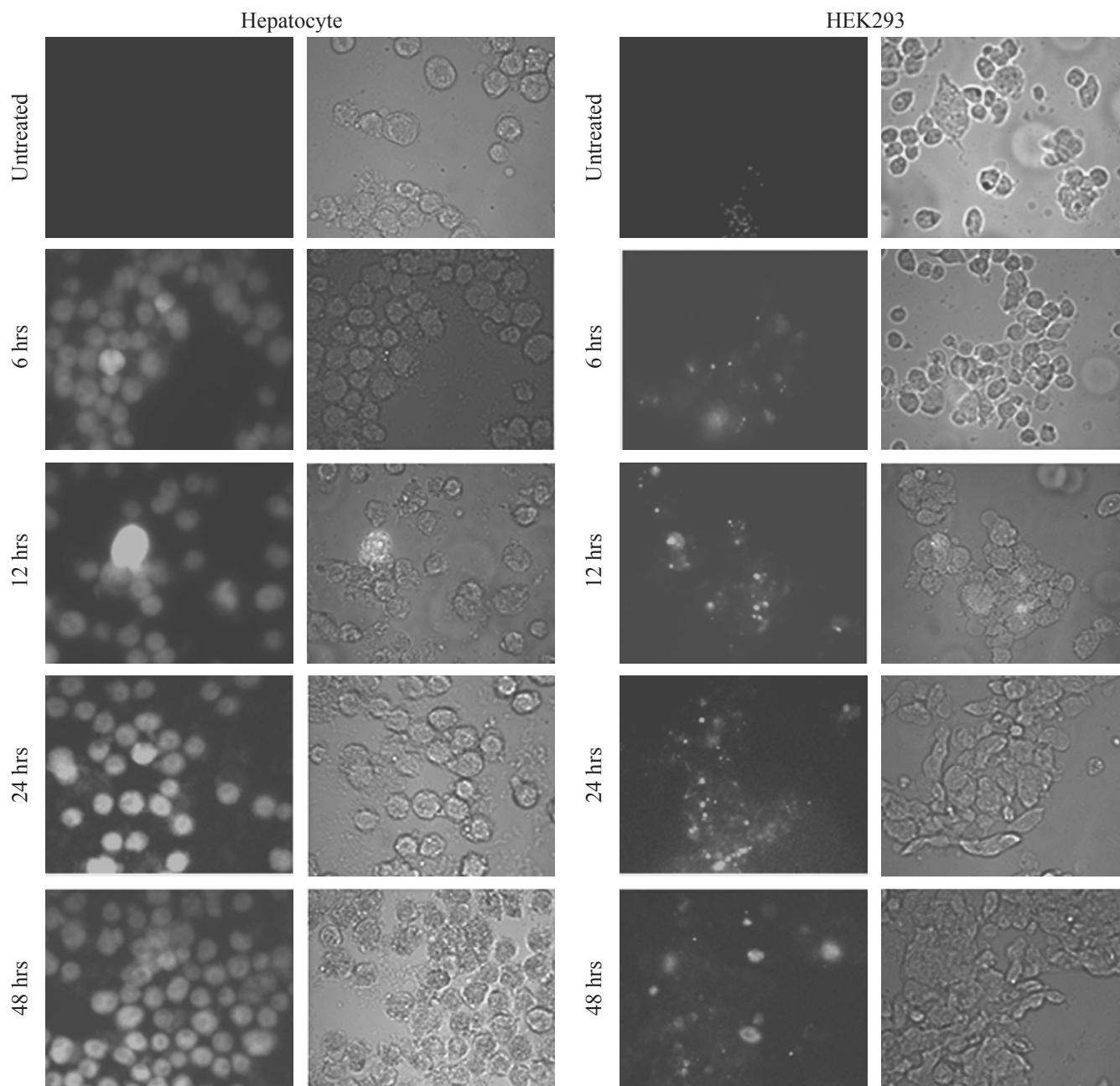
Plate 3: *In vitro* uptake of TEX615-siRNA by Liver cell transfected with Lipofectamine. (a) One day primary cultures of hepatocytes (b) One day cultures of HEK293 cells

agent (Invitrogen Inc., Carlsbad, CA) as previously described (McMahon et al., 2009, Zhang et al., 2010) and they harvested cells at 24 hours 48 hours and 72 hours after transfection, similar to the time points used in our study. McMahon et al. (2009) transfected normal human lymphoblastoid cells with αIISp siRNA or non-silencing siRNA using Lipofectamine 2000 Transfection Reagent (Invitrogen) and harvested cells at 24 and 48 hours after transfection. This study also found an optimum time scale in transfection of hepatocytes.

Recently Park et al. (2011) tested several liposome-based trans-fection reagents Metafectene Pro, Lipofectamine 2000 and Targefect in mouse primary hepatocytes for gene silencing and functional studies. They showed that transfection efficiency with one of the most recently developed formulations, Meta-fectene Pro, is high with plasmid DNA (>45% cells) as well as double stranded RNA (>90% with siRNA or microRNA). In addition, negligible cytotoxicity was present with all of these nucleic acids, even if cells were incubated with the DNA:lipid complex for 16 hours. They targeted two liver expressed genes, Sterol Regulatory Element-Binding Protein-1 and Fatty Acid
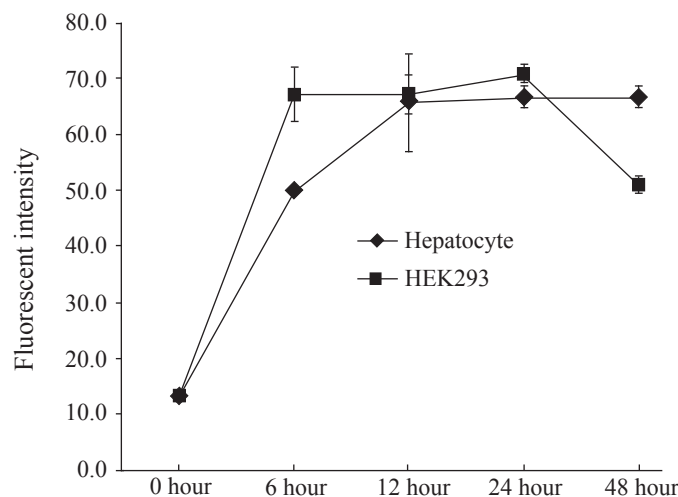
Figure 3: Quantification of fluorescent intensity of TEX 615 dye in hepatocyte and HEK293 cell lines

Binding Protein 5 using plasmid-mediated short hairpin RNA expression. In addition, similar transfection conditions were used to optimally deliver siRNA and microRNA. They identified a lipid-based reagent for primary hepatocyte transfection of nucleic acids currently used in molecular biology laboratories. The conditions described here can be used to expedite a large variety of research applications, from gene function studies to microRNA target identification.

Nguyen et al. (2003 and 2005) successfully transfected PGL3 plasmid in rat and mouse primary hepatocyte cultures using lipofectamine and another cationic liposomal vector TFL-3. Our results are supported by findings of Nguyen et al. (2003, 2005) and Park et al. (2011) that successful lipofections with cationic liposomal vector in rat and mouse primary hepatocytes were observed for all plasmid DNA tested, and the efficiencies were superior to that of commercially available Lipofectamine under there experimental conditions. The degree and rate of gene expression were dependent on incubation time prior to lipofection as well as on the density of the cells per dish, but this relationship did not hold for the amount of gene delivered. These results agree with our findings that 500 nanogram plasmid was optimum as compared to as high as 8 microgram PGL3 plasmid. We also found that lipofectamine can deliver TEX615 dye labeled siRNA, pMAX-GFP plasmid, and PGL3 plasmid as early as 6 hours in primary hepatocytes in culture.

4. Conclusion

We conducted studies to validate the *in vitro* lipofectamine platform for siRNA delivery as well as plasmid vectors to hepatocytes. Our results demonstrate a near 100% transfection efficiency of a labeled control siRNA and luciferase or GFP expressing plasmids. Our results indicate that while lipofectamine has higher transfection efficiency in HEK293 cell lines as compared to jetPEI-Hepatocyte in hepatocytes. Our findings demonstrate a promising and efficient siRNA delivery system enabling their potent application in perturbing gene regulatory networks in hepatocytes.

5. Acknowledgements

6. Reference

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