OZB Highlights

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Gene Silencing: Success stories

Summary :

Gene knockdown in hESC (Human Embryonic Stem Cells) with Lullaby®
A. Barroso-delJesus *et al.* The Nodal inhibitor Lefty is negatively modulated by the microRNA miR-302 in human embryonic stem cells.
FASEB J. 2011 May; 25(5):1497-508

siRNA delivery into monocyte-derived macrophages with SilenceMag
Kadiu *et al.* Macrophage endocytic trafficking of antiretroviral nanoparticles.
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Gene silencing in hippocampal neurons cells using SilenceMag transfection
J.C Gant *et al.* Disrupting function of FK506-binding protein 1b/12.6 induces the Ca^{2}+-dysregulation aging phenotype in hippocampal neurons.
J Neurosci. 2011 Feb 2;31(5):1693-703

OZ BIOSCIENCES
The art of delivery systems

Gene knockdown in hESC (Human Embryonic Stem Cells) with Lullaby®

FASEB J. 2011 May; 25(5):1497-508

The Nodal inhibitor Lefty is negatively modulated by the microRNA miR-302 in human embryonic stem cells.

A. Barroso-delJesus et al.

MicroRNA (miRNA) are small RNA molecules, ~22 nt long which function to block the translation and enhance the decay of target mRNA. miRNA have been shown to be important in early development and maintenance of human embryonic stem cells (hESC) where their principal function is the regulation of self renewal vs differentiation. Even if overall miRNA expression is relatively low as compared with human adult tissues, the miR-302-367 cluster displays high level intracellular expression in hESC. Nevertheless, the physiological role of specific miRNA-mRNA interactions remains largely unknown.

In this study, A. Barroso-delJesus et al aimed at dissecting putative miR-302-367 mRNA targets in hESC potentially relevant for hESC biology. hESC lines HS181 and RUES2 were cultured on Matrigel-coated flasks in human foreskin fibroblast-conditioned medium supplemented with 8 ng/mL of bFGF; medium was changed daily before transfection. **Lullaby® siRNA transfection reagent** was used in this study with high efficiency for transfecting anti-miR oligonucleotides in human Embryonic Stem Cells. In the first experiment authors transfected plasmid DNA coding for sensors for miR-302a, 302d and miR-307 and showed a 90% decrease in luciferase activity. When using **Lullaby®** for co-transfecting the corresponding anti-miR, luciferase activity was fully restored, confirming the complete and specific blockage of the miRNA activity (figure 1).

Depletion of miR-302s and miR-307 was achieved in hESC by transient transfection and 24 h later, microarray experiment was performed. Whereas knock-down of either miR rendered minor changes in global gene expression, comparison with other embryonic cell lines analysis pointed out a consistent pattern of regulation. In order to validate bioinformatic data, the authors focused on two targets involved in the TGF /Nodal signalling, PAI1 and Lefty, which play an important role in embryonic development and cell fate decision. Two different concentrations of anti-miR (20 and 200 mM) were transfected with **Lullaby®** and 24 or 48 h later mRNA levels for Lefty and PAI-1 were monitored by qRT-PCR. The microarray-predicted target Lefty was clearly validated during this experiment; PAI-1 only showed a slight up-regulation only 24 h after transfection.

Then, the authors investigated the effect of miR-302s transient depletion in hESC at the protein level. Total cell lysates were prepared 24 h after transfection of hESC with anti-miR-302. A significant increase of Lefty proteins was detectable as a result of miR-302s depletion. However, PAI-1 protein remained unchanged confirming the qRT-PCR results. In the following experiments, authors found that Lefties but not Nodal genes were post-transcriptionally targeted by miR-302s. Moreover, the miR-302 family negatively modulated level of Lefties during early differentiation demonstrating that miR-302s could be proposed as upstream regulators of the TGFb/Nodal signalling pathway in hESCs. Altogether, the findings suggest that Lefty is negatively modulated by miR-302s in hESC which plays an important role in maintaining the balance between pluripotency and germ layer specification.

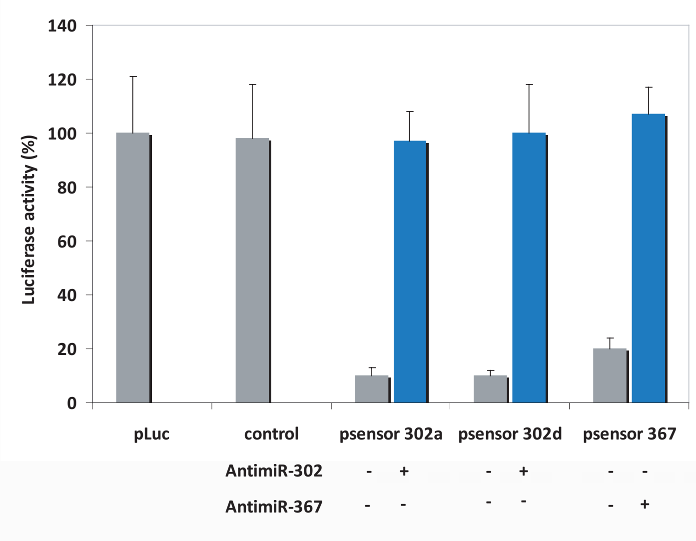


Figure 1: Functional validation of anti-miR in hESC.
hESC were transfected with plasmid sensor in presence (+) or absence (-) of their corresponding anti-miR 302 or 307. Luciferase activity was monitored and values were expressed as a % of pLuc activity.

Other successful Lullaby® transfections:
- *Primary human dermal fibroblasts*: Garinis GA et al. Nature Cell Biology. 2009; 11:604-618.
- *Primary human embryonic stem cells*: Xu N. et al. Cell. 2009; 137:1-12.
- *HeLa cells*: Pothof et al. EMBO. 2009; 28:20920-2099.
- *NRK*: K. Boeckeler et al. J Cell Science 2010, 123, 2725-32.
- *B16-F10 cells* (metastatic melanoma): Noreen F. et al. Oligonucleotides. 2009; 19:169-178.
- *HeLa cells*: Ruepp MD. et al. Nucleic Acids Res. 2010 November; 38(21): 7637–7650.

siRNA delivery into monocyte-derived macrophages with SilenceMag

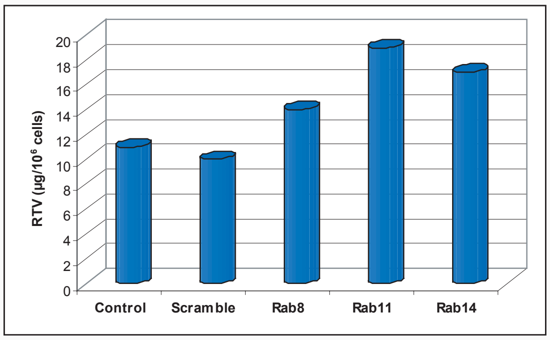
Nanomedicine, 2011 Mar 21. [Epub ahead of print].

Macrophage endocytic trafficking of antiretroviral nanoparticles.

Kadiu et al.

The successes of antiretroviral therapies (ART) for treating HIV-infected people are often limited by unfavourable pharmacokinetic and biodistribution properties of the drugs as well as a restricted access to some viral sanctuaries. Cell-mediated drug delivery is investigated as a promising alternative for overcoming these issues. Previous studies have shown that **monocyte-derived macrophages (MDMs)** can be used as cell-carriers for crystalline antiretroviral nanoparticles (nanoART). NanoART-laden MDMs are able to cross biological barriers, deliver drugs to infected tissues and drastically reduce viral replication. However, a better understanding of delivery mechanisms, thorough characterization of the subcellular distribution of the drug particles within MDM is needed.

Kadiu et al. tracked nanoART from its initial uptake by MDMs to its intracellular trafficking and release using Ritonavir particles as model. They showed that following clathrin-dependent endocytosis, drug particles undergo sorting into a recycling pathway, thereby avoiding lysosomal degradation. Interestingly, nanoART trafficking pathway is parallel to that observed for HIV particles. Transmission electron microscopy confirmed uptake of the particles into distinct cytoplasmic vesicles. Brilliant Blue-labelled nanoART where then used for subcellular distribution analysis, and stained endocytic compartments were collected and analyzed by mass spectrometry. These experiments revealed that nanoART are primarily sorted into early and recycling endosomes (EE (24%) and RE (22%)).



To validate the recycling pathway for nanoART trafficking, proteins involved in endocytic trafficking such as Rab8, 11 and 14 were knock down by siRNA **(figure 1)**.

To this end, Human monocytes obtained by leukapheresis were purified by counter-current centrifugal elutriation. Monocytes were cultivated 7 days to induce differentiation to macrophages at a concentration of 1 x 10^6 cells/mL in DMEM supplemented with 10% heat-inactivated pooled human serum, 1% glutamine, 50µg/mL gentamicin, 10 µg/mL ciprofloxacin and 1000 U/mL of macrophage colony-stimulating factor (MCSF). **Monocytes-derived macrophages** were then cultured at 2 x 10^6 per well and transfected with siRNA targeting the Rab proteins using **SilenceMag reagent**, an optimized magnetic nanoparticle formulation designed to achieve high siRNA transport and efficient protein expression knockdown.

After 72h, confocal microscopy confirmed that the distribution of nanoART in siRNA-treated MDMs was considerably altered compared with untreated MDMs.
In particular, Rab11 and Rab14 siRNA-treated cells retained more drugs, and thus released fewer drugs into the media than untreated and Rab8 siRNA-treated MDMs.
The data altogether confirmed endocytic recycling routes in both intracellular trafficking and release of nanoART. Finally, nanoART released from MDMs were shown to retain their full antiretroviral activity, with a small dose of formulated drug being able to completely suppress viral replication while an equivalent amount of the free drug had no effect.
MDMs were therefore considered as a promising carrier to deliver drugs to viral infection sites.

Figure 1:
Quantitation of Ritonavir (RTV) in cells after silencing of Rab8, Rab11 and Rab14 proteins with SilenceMag.

Gene silencing in hippocampal neurons cells using SilenceMag transfection reagent

J Neurosci. 2011 Feb 2;31(5):1693-703

Disrupting function of FK506-binding protein 1b/12.6 induces the Ca^{2+}-dysregulation aging phenotype in hippocampal neurons.

J.C Gant et al.

With aging, multiple Ca^{2+} associated electrophysiological processes are increased magnitude in hippocampal pyramidal neurons. Ca2+ dysregulation correlates with reduced neuronal excitability/plasticity and impaired learning/memory and has been proposed to contribute to unhealthy brain aging and Alzheimer's disease.
However little is known about underlying molecular mechanisms.
In cardiomyocytes, FK506-binding protein 1b/12.6 (FKBP1b) binds and stabilizes RyR2, an intracellular calcium channel, in the close state inhibiting Ca2+ release.
In a previous study, using microarrays data, the authors found that hippocampal Fkbp1b expression levels are down-regulated with aging in hippocampus of rats (Kadish et al, 2009).

In this study, J.C. Gant et al. tested whether disrupting FKBP1b function in hippocampal neurons of young animals might be sufficient to induce the aging phenotype by destabilizing Ca^{2+} homeostasis.

Primary mixed hippocampal neurons-astrocytes cell cultures from fetal pup tissue (embryonic day 18) were suspended in MEM to a final concentration of 3-5 x 10^5 cells/mL and 1 mL was added to poly-L-lysine-coated 35 mm culture dishes containing 1 mL MEM with 10 % fetal bovine and 10 % horse serum. An optimized feeding schedule was followed to maintain neurons for long periods of culture (Clodfelter et al, 2002).
Most of experiments were performed at days 7-8 in vitro.

The authors first tested the effects of Fkbp1b down-regulation mediated by siRNA on the L-type voltage-gated Ca^{2+} channel (L-VGCC) activity, one of the primary components of aging-related Ca^{2+} dysregulation.
8 nM of either siRNA directed against Fkbp1b mRNA (siFkbp1b) or non-targetting siRNA were mixed with 4 µL of **SilenceMag** beads in 200 µL of serum-free supplemented MEM at room temperature for 20 min and were then added to hippocampal cell culture to a final volume of 2 mL.
After a 15 min incubation time on a magnetic plate, cells were incubated 96 h in a 37°C cell culture incubator before current recording and qPCR analysis.
In neurons transfected with **SilenceMag** and siFkbp1b, VGCC current density was significantly enhanced versus controls **(figure 1)**.
In parallel, real-time qPCR confirmed that siFkbp1 transfection treatment achieved significant knock-down of cell culture Fkbp1b mRNA.

In the following experiments, authors first confirmed the role of FKBP1b in vitro by treatment with rapamycin, an immunosuppressant drug that binds and displaces FKBP1b and then, they assessed FKBP1b function in vivo by silencing using adeno-associated virus vector. Taken together the results showed that disruption of FKBP1b/12.6 function induced key components of aging phenotype of Ca^{2+} dysregulation in young hippocampal pyramidal neurons, implicating for the first time FKBP as candidate molecular mechanism in unhealthy brain aging.

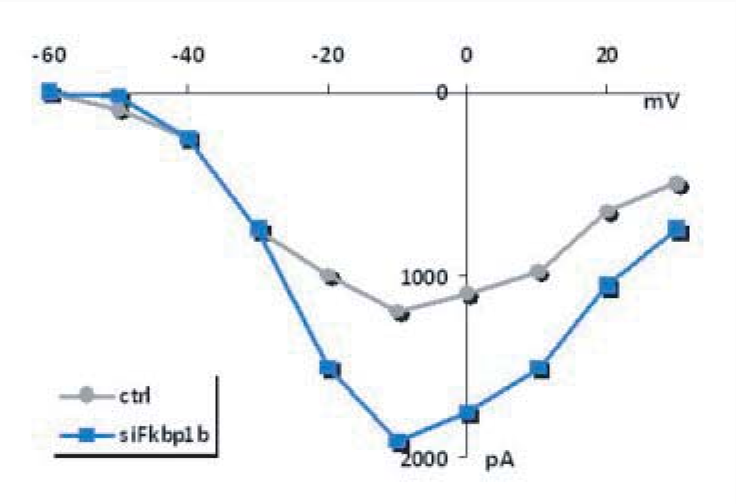


Figure 1: SilenceMag induced knock-down of FKBP1b increased Ca^{2+} channel current in cultured hippocampal neurons. Curves show mean I-V relationship for control and siRNA/SilenceMag treated neurons. Neurons transfected with siFKbp1b/SilenceMag for 96 h (blue curve) induced enhancement of VGCC current.

Other successful SilenceMag applications:
- *HUVEC & HMEC1*: S.Simoncini et al. Circ Res. 2009, 104(8):925-7
- *Neurons*: G. de Lartigue et al. Endocrinology. 2010, 151(8):3589-3599
- *C2C12*: Y.Tajika et al. Acta Histochem.Cytochem. 2010, 43 (4):107–114
- *MC3T3-E1 Osteoblasts*: F. Zhang et al. Biochimie. 2011 Feb;93(2):296-305
- *Monocyte-derived macrophages*: Kadiu et al. Nanomedicine. 2011 Mar 21. [Epub ahead of print]