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Neurobasal media facilitates increased specificity of siRNA-mediated knockdown in primary cerebellar cultures

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HIGHLIGHTS

- Accell siRNAs was tested in cerebellar granule neurons in two media.
- siRNAs delivered in Accell Delivery media caused off-target effects.
- Specific downregulation of target was obtained in Neurobasal™ media.
- The off-target effect was not a secondary response to downregulation of target.

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ABSTRACT

Background: Efficient and specific knockdown of proteins in post-mitotic cells such as differentiated neurons can be difficult to achieve. Further, special care must be taken to maintain the health of neurons in vitro. We wanted to achieve knockdown in primary cerebellar granule neurons, which can be effectively grown in Neurobasal™ media.
New method: We tested the efficiency of siRNA from the Accell range from Dharmacon™ when delivered in Neurobasal™ media in contrast to the recommended Accell Delivery media provided by the manufacturer.
Results: We observed a more specific knockdown of target in Neurobasal™ media, than in Accell Delivery media when using cerebellar granule neurons. Transfection efficiency and cell viability was comparable between the two media.
Comparison with existing methods: Delivery of siRNA in Neurobasal™ media facilitates increased specificity of the knockdown compared to delivery in Accell Delivery media. The off-target effect observed in Accell Delivery media was not a secondary biological response to downregulation of target, but rather a mixture of specific and non-specific off-target effects.
Conclusions: Specific knockdown of target can be achieved in primary cerebellar granule cells using Accell siRNAs in Neurobasal™ media. This method ensures specific knockdown in post-mitotic neurons without the need for biosafety level 2 laboratories, additional reagents, or instruments needed by other transfection

1. Introduction

Knocking down a target protein using RNA interference (RNAi) is a powerful tool for understanding protein function and the molecular mechanism underlying a given observation. RNAi can readily be achieved in dividing cell lines by the introduction of small interference RNA (siRNA) (Elbashir et al., 2001). However, differentiated neurons and other post-mitotic cells are more challenging than dividing cells to transfect with nucleic acids (Washbourne and

Abbreviations: CGNs, cerebellar granule neurons; Ara-C, cytosine β-D-arabinofuranoside hydrochloride; NBM, Neurobasal™ media.

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McAllister, 2002; Karra and Dahm, 2010; Perez-Martinez et al., 2011). Not only it is more difficult to achieve an efficient transfection in differentiated neurons, but dissociated neurons are also more sensitive to changes in their microenvironment, such as temperature, pH, or physical stress, all of which might be changed during the transfection procedure. This will result in a low percentage of neurons surviving the transfection (Karra and Dahm, 2010). High-efficiency transfection of post-mitotic neurons was first achieved in 1988 by employing a replication-defective viral vector (Geller and Breakefield, 1988). Since then, a range of different methods for transfecting post-mitotic neurons has been developed, each with their own advantages and disadvantages (for review see (Craig, 1998; Washbourne and McAllister, 2002)). For instance, viral infection facilitates high-efficiency transfection, with stable and long-term downregulation, but it is labor-intensive to produce viral vector particles and the procedure requires a higher level of biosafety than otherwise needed. For every experiment, it is thus worthwhile to consider the purpose of the transfection and to choose a method that is "fit for purpose". In our case, we wanted to specifically downregulate one target gene at a time for a period of up to 7 days in dissociated cultures of cerebellar granule neurons (CGNs). We chose to use siRNA from the Accell range from Dharmacon™ for two reasons: 1) this method facilitates transfection of post-mitotic neurons without the need for biosafety level 2 laboratories, additional reagents, or instruments, and 2) we did not need to achieve long-term downregulation of the target genes. The Accell siRNA system consists of the modified siRNA, which are introduced into the cells in the presence of Accell Delivery media. The Accell Delivery media is an enriched serum-free media, but it is not specifically developed for neuronal culturing. It is well known that special care must be taken when culturing neurons in vitro (Romijn, 1988). The most commonly used media for culturing dissociated neuronal cultures: Neurobasal™ media (NBM) supplemented with B27 was developed for culturing hippocampal neurons (Brewer and Cotman, 1989; Brewer et al., 1993), but has been applied for a range of different neuronal cultures (Craven et al., 1999; Ullian et al., 2001; Passafaro et al., 2003; Tomita et al., 2004; Sans et al., 2005; Stellwagen and Malenka, 2006), including CGNs (Brewer, 1995; Pankratova et al., 2012). Although CGNs can be grown in a relatively simple media (Messer et al., 1981), they are specifically sensitive to changes in K+ ion concentration (Kingsbury et al., 1985; Mogensen et al., 1994; Mellor et al., 1998; Suzuki et al., 2005). It has also been found that the survival of CGNs is even better in NBM-A, which contains higher concentration of NaCl than NBM (Pankratova et al., 2012). For these reasons, we decided to compare the efficiency of gene knock down by Accell siRNA in Accell Delivery media, or in NBM-A depleted of Bovine Serum Albumin (BSA).

2. Materials and methods

2.1. Primary cultures of cerebellar granular neurons

Primary CGN cultures were prepared from 7 to 9 BALB/cJBomTac (Taconic, Denmark) pups at postnatal day six, essentially as described by Schousboe et al. (1989). Cerebellum was isolated from a maximum of two pups at a time, the meninges removed on ice in a dissection buffer containing Krebs-Ringer buffer (Substrate-department, Faculty of Health Science, University of Copenhagen), BSA (Sigma-Aldrich, St. Louis, MO, USA), $MgSO_4$ (RegionH Apoteket), and HEPES buffer (Gibco, Life Sciences), and the remaining tissue was mechanically and enzymatically homogenized by chopping and trypsination. Cells were then washed in a buffer containing trypsin inhibitor and DNase I (Sigma-Aldrich, St. Louis, MO, USA), centrifuged briefly at 100 rpm to stratify cells, and the uppermost layers were transferred to Krebs-Ringer buffer supplemented with BSA (Sigma-Aldrich, St. Louis, MO, USA), $MgSO_4$ (RegionH Apoteket), HEPES (Gibco, Life Sciences), $CaCl_2$ (RegionH Apoteket). Upon centrifugation for 10 min at 700 rpm, the pelleted cells were resuspended in NBM-A (Gibco, Life Sciences) supplemented with B27 (Gibco, Life Sciences), BSA (Sigma-Aldrich, St. Louis, MO, USA), Glutamax (Gibco, Life Sciences), and HEPES (Gibco, Life Sciences). Cells were seeded at a density of $4 \times 10\hat{5}$ cells/mL in 24-well plates coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C, 5% CO_2. 24 h after seeding of cells, cytosine β-D-arabinofuranoside hydrochloride (Ara-C) (Sigma-Aldrich, St. Louis, MO, USA) was added to the media to a final concentration of 10 μM to inhibit the growth of glial cells.

2.2. siRNA for primary cultures of cerebellar granular neurons

The siRNAs against *DNMT1*, *DNMT3a*, *DNMT3b* and *PPIB* were bought as a pool of four siRNAs targeting the same gene in different sites, called SMARTpool, from the Accell range by Dharmacon™, part of GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom (See Table 1). Negative control siRNA, "Accell Non-targeting #1" was designed, modified and microarray-confirmed by Dharmacon™ to have minimal targeting of known genes in mouse cells. We validated that the siRNAs against *DNMT1* had no targets in transcripts of *DNMT3a* and *DNMT3b*, by using the less restrictive BLAST algorithm blastn from ncbi genome aligning tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The siRNAs were diluted in the supplied 5× siRNA buffer diluted in RNase-free water, according to the manufacturer's instructions, aliquoted and stored at −20 °C.

2.3. Delivery of siRNA in primary cultures of cerebellar granular neurons

Cerebellar granular cultures were treated with 1 μM siRNA, SMARTpool or non-targeting siRNA control, 24 h after seeding cells. The growth media was exchanged with preheated and CO_2-equilibrated NBM-A media (Gibco, Life Sciences) supplemented with B27 (Gibco, Life Sciences), Glutamax (Gibco, Life Sciences) and HEPES (Gibco, Life Sciences) or Accell Delivery media (Dharmacon™, GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), both containing 10 μM Ara-C and 1 μM siRNA or without siRNA for untreated condition. Neuronal cultures were incubated at 37 °C, 5% CO_2 for 72 h in the siRNA-containing media, before assessing gene expression levels by real-time quantitative PCR. Data are obtained from experiments done in parallel: Primary CGNs from the same batch of preparation were divided into two groups, either receiving the siRNAs in Accell Delivery media, or in NBM-A media.

2.4. N2a cell culturing and delivery of siRNA in cell line

The murine neuroblastoma cell line N2a was obtained from ATCC. Undifferentiated N2a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Biowest, Nuaillé, France), supplemented with 10% Fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), and 0.5% penicillin-streptomycin (Lonza Ltd, Basel, Switzerland). 24 h after seeding the cells were transfected with siRNA against *DNMT1* or negative scramble control (ThermoFischer Scientific, Waltham, MA USA). Transfection was achieved using Lipofectamine® 2000 Transfection Reagent according to the manufacturer's instruction (Invitrogen, ThermoFischer Scientific Waltham, MA USA). After transfection the N2a cultures were incubated at 37 °C, 5% CO_2 for 72 h in normal growth media, before assessing gene expression levels by real-time quantitative PCR.