A peptidomimetic siRNA transfection reagent for highly effective gene silencing†

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RNA interference (RNAi) techniques hold forth great promise for therapeutic silencing of deleterious genes. However, clinical applications of RNAi require the development of safe and efficient methods for intracellular delivery of small interfering RNA (siRNA) oligonucleotides specific to targeted genes. We describe the use of a lipitoid, a cationic oligopeptoid–phospholipid conjugate, for non-viral transfection of synthetic siRNA oligos in cell culture. This peptidomimetic delivery vehicle allows for efficient siRNA transfection in a variety of human cell lines with negligible toxicity and promotes extensive downregulation of the targeted genes at both the protein and the mRNA level. We compare the lipitoid reagent to a standard commercial transfection reagent. The lipitoid is highly efficient even in primary IMR-90 human lung fibroblasts in which other commercial reagents are typically ineffective.

Introduction

The potential of synthetic siRNA oligonucleotides as a therapeutic modality is increasingly evident. These 21–23 nucleotide long nucleic acids exert a specific gene silencing effect by activating the cell's own RNAi mechanism,[1,2] and can lead to complete knock-down of the targeted gene.[3] However, delivery of these molecules into diverse cell types, such as primary cells, remains a critical challenge for RNAi-based studies.[4] Delivery methods that will increase the efficiency of siRNA uptake are therefore required. A variety of such methods, ranging from electroporation to commercial chemical delivery reagents, are currently being evaluated for the transfection of siRNA. The most efficient delivery to date has been achieved with viral transfection vectors, in which the viral genetic material is engineered to carry a coding region for the siRNA. However, construction of a separate vector for each siRNA may be impractical for large-scale applications, and the use of viral vectors has elicited substantial safety concerns. Consequently, there is a clear need for a non-viral reagent that will achieve fast and efficient transfection with low cytotoxicity, and enable administered siRNA molecules to exhibit their potential for specific gene knock-down in a wide variety of cells.

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In previous studies, cationic oligomers comprised of *N*-substituted glycine units were found to have good capabilities for transfection of plasmid DNA,[5] suggesting the possible utility of these "peptoids" in transfection of siRNA. The *N*-substituted amide groups provide for a high resistance to proteolytic degradation,[6] which is a desirable attribute in the design of improved transfection reagents.[7] Peptoids can be synthesized *via* a robust solid phase method to generate monodisperse products incorporating a specific sequence of monomers with diverse functional groups.[8] The modular nature of peptoid synthesis creates a practical chemical platform for the assembly of molecules that can be used in a range of biomimetic studies and biomedical applications.[9]

Peptoids containing cationic side chains are known to penetrate the cell membrane[10] and associate with DNA, leading to condensation of plasmid DNA into 60 nm–100 nm sized toroids with an overall positive charge.[11] These complexes are thought to sequester the nucleic acid through electrostatic interactions and thus protect the plasmid DNA from nucleases prior to entry into the cytoplasm. Peptoids with a trimer repeat of a hydrophilic–hydrophobic–hydrophobic sequence showed consistently better DNA transfection efficiencies as compared to scrambled peptoid sequences. The performance of the peptoids was further improved by conjugating a phospholipid group to the oligopeptoid scaffold, thereby generating "lipitoids". Some of the lipitoids showed diminished cytotoxicity and increased DNA transfection efficiency in comparison to commercially available products such as lipofectine, lipofectamine and polylysine.[11] The lipid conjugate is therefore deemed a moiety that can interact effectively with the cell membrane to facilitate uptake of the plasmid into the cell.

In this study, we utilize a lipitoid, DMPE–$(NaeNmpeNmpe)_3$, for siRNA delivery into a variety of cell types. The molecule consists of a peptoid possessing a trimeric sequence repeat conjugated to DMPE, a phospholipid with

two fatty acyl chains. Addition of the phospholipid was determined to enhance the capability of the peptoid to transfect plasmid DNA and chimeric antisense oligonucleotides.[11,12] To determine the suitability of this molecule as an effective siRNA transfection agent, its performance was assessed in cell culture using common immortalized cell lines as well as primary cells.

Results

Optimization of conditions for siRNA transfection

The lipitoid reagent DMPE–$(NaeNmpeNmpe)_3$ (Fig. 1) was prepared as previously described.[12–14] Briefly, the peptoid oligomer was synthesized on a solid phase support by standard submonomer addition protocols, followed by DMPE conjugation. The lipitoid was then cleaved off the resin, purified by HPLC and characterized by LC-MS analysis.

To test the viability of the lipitoid DMPE–$(NaeNmpeNmpe)_3$ as a transfection agent for siRNA rather than DNA, it was evaluated in a series of optimization experiments in HeLa human cervical cancer cells. Optimizations included varying the lipitoid : siRNA charge ratios (positive charges on lipitoid : negative charges on siRNA) from 1 : 1 to 4 : 1, incubating the siRNA–lipitoid complexes for periods of 1–6 h, and varying the siRNA and/or lipitoid concentrations. The most efficient downregulation of the targeted gene was observed at a charge ratio of approximately 3 : 1 for an incubation time of 1.5 h (data not shown). Similar results were obtained in T98G human brain glioblastoma cells. These optimal parameters were employed in all further transfection experiments with the lipitoid DMPE–$(NaeNmpeNmpe)_3$.

In order to determine the optimal siRNA concentration, initial transfection experiments with siRNA concentrations varying between 10 nM and 100 nM were performed in T98G cells with siRNA against skp2 (s-phase kinase-associated protein 2). These concentrations are generally employed in transfections with commercial reagents.[15] Western Blot analysis with specific antibodies against skp2 and the loading control cul1 were performed 48 hours after transfection and indicated that the protein downregulation was dependent on siRNA concentration (Fig. 2a). Quantification of Western Blots revealed that a concentration of 100 nM led to 90% downregulation, while the use of 50 nM siRNA yielded 82% downregulation (Fig. 2b). It is desirable to use a minimal amount of siRNA since high siRNA concentrations can lead to unwanted off-target effects. Consequently, siRNA concentrations were kept at 50 nM throughout the subsequent experiments, except in experiments comparing the lipitoid to Oligofectamine, where 100 nM siRNA was used for consistency (see below).

Transfection of different siRNA sequences with lipitoid and RNAi mediated gene downregulation

To assess the suitability of the lipitoid DMPE–$(NaeNmpeNmpe)_3$ as a generally applicable transfection reagent, transfection experiments with siRNA against skp2, cdc20 (cell division cycle protein 20) and cdh1 (Fizzy-related protein homolog) were performed in cell culture (see Supplementary Table 1 for sequences).† Experiments were performed in T98G cells. In all experiments, more than one siRNA sequence was used for each targeted gene, and siRNA concentrations were 50 nM. LacZ siRNA was used as negative control. Cells were lysed 48–52 h after transfection and specific protein levels were determined by Western Blot analysis with specific antibodies against the targeted proteins and cul1 as a loading control. The substantial decrease in the amount of the targeted proteins as compared to the LacZ siRNA treated control cells indicates that the lipitoid delivers the siRNA into the cells, causing highly efficient downregulation of all three targeted proteins. A >80% decrease in mRNA levels as determined by quantitative RT-PCR confirmed that the decrease in the targeted protein expression was an RNAi effect (Fig. 3).

A standard MTT based assay was used to demonstrate that there was no cytotoxicity associated with either the lipitoid alone or the transfection complexes of siRNA and lipitoid. The >90% cell viability in both cases revealed that there is virtually no cytotoxicity caused by these high transfection efficiencies

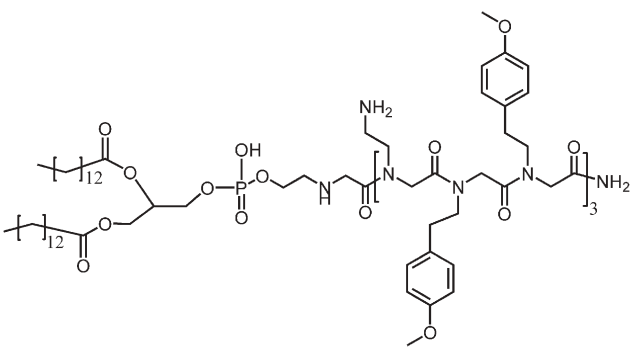

Fig. 1 Chemical structure of the lipitoid transfection reagent DMPE–(NaeNmpeNmpe)3 consisting of a phospholipid conjugated to a cationic peptoid backbone (DMPE, dimirystoyl phosphatidyl–ethanolamine; Nae, *N*-(2-aminoethyl)glycine; Nmpe, *N*-*p*-methoxyphenethylglycine).

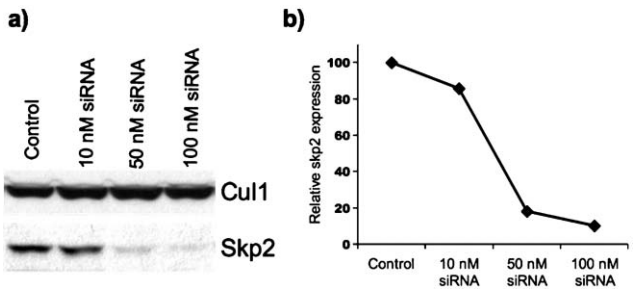

Fig. 2 Lipitoid transfects siRNA and causes concentration dependent downregulation of the targeted protein. Varying concentrations (10 nM–100 nM) of skp2 siRNA2 were tested in T98G cells. Transfection experiments were done with the lipitoid DMPE–(NaeNmpeNmpe)3 as transfection agent and LacZ siRNA as the control siRNA (50 nM). (a) Western Blot analysis with specific antibodies against skp2 and the loading control cul1 was performed 48 h after transfection. (b) Skp2 amounts as quantified from the Western Blot.

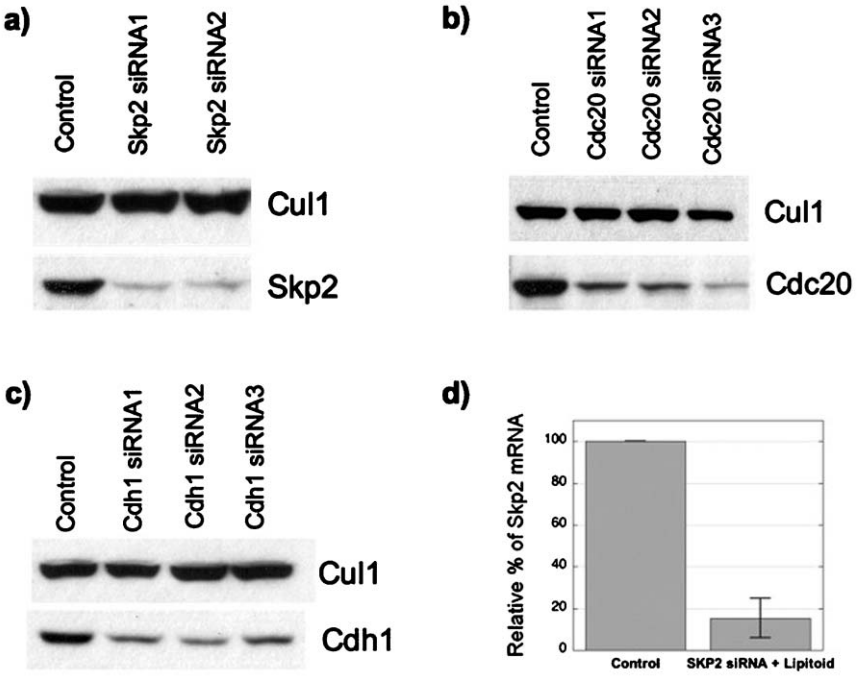

Fig. 3 Transfection of siRNA leads to effective downregulation of target proteins through RNA interference. Transfection experiments were carried out in T98G cells using three different protein targets. A variety of siRNA sequences (50 nM) for each target protein were tested individually. Transfections were carried out with the lipitoid DMPE–(NaeNmpeNmpe)3 as the transfection reagent and LacZ siRNA as negative control. Targeted protein levels were determined 48 h after transfection. Western Blot analyses with specific antibodies against the targeted proteins and the loading control cul1 show effective downregulation of the target proteins (a) skp2, (b) cdc20 and (c) cdh1. (d) Skp2 mRNA levels in T98G cells treated with 50 nM skp2 siRNA and lipitoid were evaluated by extracting whole RNA from treated cells 48 h after transfection, followed by real-time RT-PCR with specific primers for Skp2. The extent of Skp2 mRNA expression is plotted relative to the control (LacZ) siRNA treated cells. All mRNA was normalized to GAPDH amounts.

(data not shown). Identical results were obtained in HeLa cells. The lipitoid reagent DMPE–$(NaeNmpeNmpe)_3$ is thus an effective and non-toxic vehicle for siRNA transfection in cell culture.

Comparison to a common cationic lipid transfection reagent

After the effectiveness of the lipitoid reagent DMPE–$(NaeNmpeNmpe)_3$ was demonstrated, it was compared to a standard siRNA transfection reagent, Oligofectamine, that is widely used for siRNA transfection in cell culture. Experiments were performed in T98G cells in OptiMEM. Oligofectamine was used with 100 nM siRNA according to the manufacturer's recommendations.[15] Lipitoid–siRNA complexes were incubated with cells for 1.5 h before serum addition, while Oligofectamine–siRNA complexes were incubated for 4–6 h with a single cycle of transfection as instructed by the manufacturer's protocol. Cells were harvested after 48–52 h and assayed for protein expression levels using specific antibodies against each protein. The experiment was repeated sequentially in triplicate. Western Blot analysis demonstrated that skp2 downregulation upon transfection by skp2 siRNA in complex with the lipitoid is superior to skp2 siRNA in complex with Oligofectamine (Fig. 4). Knockdown of additional genes and additional cell lines was also evaluated (See Supplementary Material Fig. 1, 2 and 3†). These results indicated that the lipitoid produced a response that is equivalent to or superior to the extent of downregulation upon transfection in the presence of Oligofectamine.

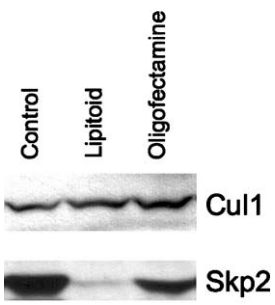

Fig. 4 Lipitoid-mediated siRNA transfection is compared to siRNA transfection with a standard transfection reagent. Skp2 siRNA2 (100 nM) was used with lipitoid or Oligofectamine as the transfection reagent. LacZ siRNA (100 nM) was used as a control. T98G cells were lysed 48 h after transfection followed by Western Blot analysis with specific antibodies against skp2 and the loading control cul1, respectively. The faint skp2 band in the lipitoid treated cells as compared to the relatively intense skp2 band in the Oligofectamine treated cells demonstrates the efficiency of lipitoid for gene silencing in complex with this siRNA.

Transfection of primary cells with the lipitoid reagent

The next goal was to investigate the effectiveness of the lipitoid reagent with IMR-90 primary fibroblasts. Like most other primary cell types, primary IMR-90 cells are resistant to chemical transfection.[16] As distinct from HeLa and T98G cells, IMR-90 are primary cells which are not immortalized or transformed, but can still be maintained in cell culture for a relatively short time before they reach senescence (~58 population doublings).[17] To ensure that results did not depend on the passage number of the cells, transfection experiments were carried out at different times over a range of passage numbers with a single transfection cycle. Experiments were repeated in triplicate using 50 nM siRNA. Western Blot analyses performed 48–52 h after transfection confirmed up to 98% downregulation of the targeted proteins skp2, cdc20 and cdh1 (Fig. 5a, 5b and 5c, respectively). RNAi was established as the mechanism of gene downregulation by quantitative RT-PCR results showing ~80% decrease in mRNA expression (representative data for skp2 is shown in Fig. 5d). The potency of the lipitoid was compared to Oligofectamine in IMR-90 cells using skp2 siRNA with both reagents. As for immortalized cells, the lipitoid was extremely efficient, causing >90% downregulation of the targeted gene, while Oligofectamine was virtually ineffective (Fig. 5e).

Discussion

siRNA oligonucleotides are promising candidates as therapeutics for a range of diseases that involve upregulation of specific genes or expression of deleterious genes.[18–21] The current challenge is to deliver siRNA with high efficiency and minimal toxicity to a range of cell types, especially to cell types such as primary cells, which are resistant to standard transfection agents.[4] Typically, chemical transfection methods that are currently employed in common cell culture are practical only in cell lines that are easily transfected. In primary cells, harsher experimental conditions such as transfecting the cells multiple times with high concentrations of siRNA or incubating the cells with the siRNA–transfection reagent complex for extended periods may lead to minor improvements in downregulation but typically at the cost of increased toxicity. Electroporation and nucleofection are usually tested as alternatives, but generally a considerable fraction of the viable cells are sacrificed with these methods. On the other hand, viral vectors offer very high transfection efficiencies but are less preferable due to safety concerns and long vector preparation times.

Transfection reagents were initially developed to facilitate the entry of plasmid DNA into cells. Although siRNA molecules are able to enter cells on their own,[22] transfection agents enhance cellular uptake and are required when efficiency is critical.[20,21] Recent studies have utilized chemically modified siRNAs that are more stable,[23–25] and/or possess a targeting entity.[18,19] These modifications have proven to be very successful; however, they need to be implemented on each of the siRNA molecules that are to be used in a specific experiment. Given the large number of genes that can be targeted and the even larger number of siRNAs

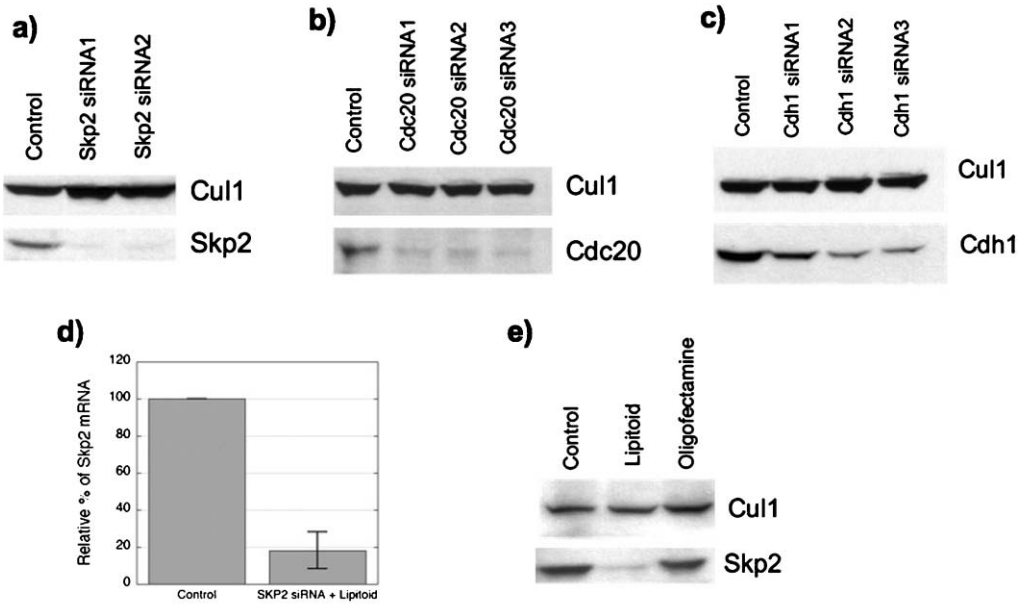

Fig. 5 Lipitoid is capable of delivering siRNA into IMR-90 primary cells. Western Blot analyses show efficient downregulation of the targeted proteins (a) skp2, (b) cdc20, and (c) cdh1, respectively. In all experiments, siRNA (50 nM) was transfected into IMR-90 primary cells using the lipitoid DMPE–(NaeNmpeNmpe)3 as a transfection reagent and LacZ siRNA(50 nM) as negative control. (d) The decrease in Skp2 mRNA levels in IMR-90 upon transfection with skp2 siRNA with lipitoid as quantified by real-time RT-PCR, confirm RNA interference effect. (e) The Western Blot demonstrates that the lipitoid transfection of skp2 siRNA was efficient and resulted in significant decrease of skp2 expression, while skp2 siRNA with Oligofectamine does not generate any quantifiable difference in the expressed skp2 amount in this cell line.

that can be used to target them, modifying each siRNA oligonucleotide individually would be laborious. To address this problem, there is a need for transfection agents that can be used readily with diverse siRNA sequences. Ideally such agents would also be multifunctional in the sense of being able to simultaneously stabilize the siRNA and deliver it efficiently.

We assess the siRNA transfection efficiency of a lipitoid reagent, a modular molecule composed of a cationic peptoid oligomer and a phospholipid moiety. Lipitoids were previously demonstrated to have DNA plasmid transfection properties; however, notwithstanding the structural similarities of RNA and plasmid DNA, the two nucleic acid species may show different behavior with respect to complex formation and condensation.[26] DNA plasmids are macromolecules that need to be condensed in order to traverse across the cell membrane of their target cells, while siRNA oligonucleotides are comparatively small molecules. Therefore it is necessary to evaluate the transfection properties and formulation requirements of DNA delivery reagents with siRNA oligomers.

An ideal transfection reagent achieves high transfection efficiency and does not cause toxicity. However, it can be challenging to achieve both of these characteristics in tandem. The lipitoid, DMPE–$(NaeNmpeNmpe)_3$, was used as a transfection reagent for delivery of siRNA oligonucleotides into HeLa and T98G cells. The resultant downregulation of the targeted genes was monitored by Western Blot analysis and quantification showed up to 98% decrease in the targeted protein levels. This effect was confirmed to arise due to RNAi mechanisms by real-time RT-PCR. The use of a variety of siRNA sequences for each targeted gene eliminated the possibility that the effect might be due to the structural characteristics of a unique siRNA sequence. Finally, the consistent results with three different genes verify that the effects are not specific to a particular gene. Cytotoxicity was found to be insignificant as demonstrated by over 90% cell viability. Therefore, the high siRNA transfection efficiencies of the lipitoid DMPE–$(NaeNmpeNmpe)_3$ and the absence of cytotoxicity for the reagent makes it suitable for biomedical studies. Most importantly, the lipitoid delivers siRNA to primary fibroblast IMR-90 cells very efficiently and causes downregulation of the targeted gene to a near-complete knockdown level. Unlike immortalized cells, primary cells are very similar to their *in vivo* counterparts. Hence, successful results with primary cells are especially important in moving from cell culture to *in vivo* applications.

Conclusions

We demonstrate the lipitoid molecule DMPE–$(NaeNmpeNmpe)_3$ to be a highly convenient reagent for use in siRNA transfection in diverse cells. The molecule demonstrated very high transfection efficiencies and was virtually non-toxic to several immortalized mammalian cell lines, such as HeLa and T98G. Transfections of siRNAs against skp2, cdc20 and cdh1 with the lipitoid were equivalent or superior to those with the commercial transfection reagent Oligofectamine. Strikingly, transfection was observed even with primary IMR-90 (human fetal fibroblast) cells, which are resistant to most commercial transfection reagents. siRNA was efficiently delivered in a single transfection to these cells, leading to decreased targeted protein and mRNA levels *via* RNA interference.

Experimental

Cell types and cell culture

Human cervical carcinoma cells (HeLa) and human brain glioblastoma cells (T98G) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) supplemented with 10% calf serum (Invitrogen) and 1% penicillin–streptomycin–glutamine (Invitrogen). Human fibroblast primary cells (IMR-90) were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin–glutamine. All cells were incubated at 37 °C at 5% CO_2.

siRNA oligonucleotides

The siRNA sequences were designed using an algorithm previously developed at Memorial Sloan-Kettering Cancer Center.[27] siRNAs were chemically synthesized using TOM-Amidites (Qiagen). Sense and antisense strands were HPLC-purified separately and checked for mass accuracy prior to annealing.(For siRNA sequences see Supplementary Table 1).†

Transfection assays

The cells were counted 24 h prior to transfection in order to achieve 60–80% cell density on the day of transfection. In a typical experiment with lipitoids, 2×10^5 cells/well were plated in a 6-well culture plate. Immediately before transfection, the medium was completely aspirated, followed by two washes with 1 ml PBS (Sigma-Aldrich) and one wash with 1 ml OptiMEM (Invitrogen) before replacement with 1.8 ml OptiMEM. The transfection cocktail was prepared by diluting the lipitoid to the desired concentration in OptiMEM and mixing with siRNA in OptiMEM, to produce 50 nM or 100 nM overall siRNA concentration, respectively. After incubating at room temperature for 10 min to allow for complex formation, 200 μl of the transfection cocktail was added to each well and the cells were incubated in a 5% CO_2 environment at 37 °C. After 1.5 h, concentrated serum was added to achieve the regular growth medium serum concentration of 10%. The cells were incubated at 37 °C for ~48 h. Oligofectamine (Invitrogen) was used according to the manufacturer's instructions.

Western blot analysis

Cells were harvested 48–52 h after transfection and were lysed in Triton X-100 buffer supplemented with protease inhibitors. Total protein content was determined using the Protein D_c assay (Bio-Rad). Proteins were separated on a 10–12% sodium dodecylsulfate–polyacrylamide gel and transferred to a PVDF membrane (Millipore). Proteins were detected by blotting the membrane with specific primary antibodies against cul1 (Zymed), skp2 (Zymed), cdc20 (Santa Cruz) or cdh1 (NeoMarker). After incubating with peroxidase conjugated secondary antibodies (Amersham Biosciences), the protein bands were visualized by chemiluminescence (ECL, Amersham

Biosciences) according to the manufacturer's protocol. Cul1 protein levels were used as an indicator of the protein amount loaded in each lane. All experiments were done in triplicate. Western Blots were quantified densitometrically using the software Quantity One (Bio-Rad).

Real-time PCR analysis

Cells were harvested 48–52 h after transfection and total RNA was extracted with RNeasy RNA isolation kit (Qiagen) following the manufacturer's protocol. Reverse transcription was carried out with 1–3 μg of RNA using Oligo dT primers and M-MLV reverse transcriptase (Promega), followed by the analysis of 0.2 μg of the resultant cDNA using specific primers (for sequences see Supplementary Table 2†) and iQSupermix (Bio-Rad) on an iCycler instrument (Bio-Rad). The comparative C_t method was used to quantify the target mRNA levels where *GAPDH* was used as an internal control for normalizing the cDNA amount in each sample.

Cytotoxicity assays

48 h after transfection, the cells were treated with the Vybrant MTT Cell Proliferation Assay Kit (Molecular Probes) following the manufacturer's protocol. Absorbance was read on a Bio-Rad Model 550 Microplate Reader at 570 nm.

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