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Gene silencing following siRNA delivery to skin via coated steel microneedles: *in vitro* and *in vivo* proof-of-concept

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Abstract

The development of siRNA-based gene silencing therapies has significant potential for effectively treating debilitating genetic, hyper-proliferative or malignant skin conditions caused by aberrant gene expression. To be efficacious and widely accepted by physicians and patients, therapeutic siRNAs must access the viable skin layers in a stable and functional form, preferably without painful administration. In this study we explore the use of minimally-invasive steel microneedle devices to effectively deliver siRNA into skin. A simple, yet precise microneedle coating method permitted reproducible loading of siRNA onto individual microneedles. Following recovery from the microneedle surface, lamin A/C siRNA retained full activity, as demonstrated by significant reduction in lamin A/C mRNA levels and reduced lamin A/C protein in HaCaT keratinocyte cells. However, lamin A/C siRNA pre-complexed with a commercial lipid-based transfection reagent (siRNA lipoplex) was less functional following microneedle coating. As Accell-modified “self-delivery” siRNA targeted against CD44 also retained functionality after microneedle coating, this form of siRNA was used in subsequent *in vivo* studies, where gene silencing was determined in a transgenic reporter mouse skin model. Self-delivery siRNA targeting the reporter (luciferase/GFP) gene was coated onto microneedles and delivered to mouse footpad. Quantification of reporter mRNA and intravital imaging of reporter expression in the outer skin layers confirmed functional *in vivo* gene silencing following microneedle delivery of siRNA. The use of coated metal microneedles represents a new, simple, minimally-invasive, patient-friendly and potentially self-administrable method for the delivery of therapeutic nucleic acids to the skin.

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Keywords

Microneedles; Coating; Lipoplex; Self-delivery siRNA; Keratinocytes; RNA interference

1. Introduction

The concept of RNA interference (RNAi) emerged in 1998 [1] and the functionality of a synthetic small interfering nucleic acid (siRNA) in mammalian cells was demonstrated in 2001 [2]. Breakthrough discoveries in recent years have led to a sustained interest in RNAi research and numerous publications have now established the potential of siRNA technology both *in vitro*, *in vivo* and in clinical trials (reviewed in [3, 4]).

The skin has a large surface area and is the most accessible organ of the body, thus lending itself to gene modification approaches [5-7]. Well controlled treatment of a confined area of the skin is possible and any genetically modified region can be monitored and biopsied for functional improvement and/or removed surgically if unwanted side effects were to occur [8]. The successful development of siRNA therapies has significant potential for the treatment of skin conditions caused by aberrant gene expression, including allergic skin diseases [9-11], alopecia [12], skin cancer [13-20], psoriasis [16], hyperpigmentation [21] and pachyonychia congenita [22, 23].

One of the most significant challenges in siRNA therapy is the effective transfer of nucleic acid across cellular membranes. This challenge is further compounded in the skin by the non-viable outermost barrier layer, the stratum corneum. Previous studies have used hypodermic needles for viable, intradermal delivery of therapeutic siRNA [22, 23]; however the significant pain associated with localised injections into diseased tissue has hindered progress to the clinic [22]. To provide a less invasive method for overcoming the stratum corneum barrier, we investigate the use of steel microneedle devices for the functional delivery of siRNA into the skin. To interact effectively with the complex multi-layer structure of the skin, microneedles, typically comprising a plurality of projections of micron-sized dimension, are designed to create micron-sized channels within the epidermal layer through which therapeutic molecules and macromolecules can be delivered [24] in a pain-free manner [25-27].

Methods of microneedle-assisted drug delivery are commonly categorised into four general approaches: (i) pre-applying solid microneedles before drug application to “punch holes” in the stratum corneum barrier through which drugs can later pass, (ii) coating drugs onto microneedles, (iii) incorporating drug into dissolving or biodegradable microneedles, and (iv) injecting drugs through hollow microneedles [28]. In previous studies, delivery of functional unmodified siRNA through intradermal injection [29] and modified self-delivery siRNA through a microneedle-based delivery system called a biodegradable protrusion array device (PAD) [30] induced silencing of reporter gene expression in the epidermis of a transgenic mouse model. Steel microneedle devices have prospective clinical advantages of simple and cost-effective manufacture with high reproducibility, biocompatibility, reliable skin puncture and sufficient loading capacity for nucleic acid therapies [31, 32]. Our aim is to evaluate the potential of stainless steel microneedles as a means to deliver surface-coated siRNA to the target region, the viable epidermis, of skin. Whilst we have recently demonstrated the utility of a similar microneedle system for plasmid DNA delivery to skin [32], this is the first study exploring the utility of this simple delivery system for siRNA delivery. The study confirms appropriate siRNA loading onto microneedles, siRNA deposition within skin and functionality, through demonstrable gene silencing *in vivo*.

2. Materials and methods

All reagents and laboratory equipment was purchased from Thermo Fisher Scientific (UK or USA) unless otherwise stated.

2.1. siRNA sequences

2.1.2 *In vitro* studies—The siRNA molecules used were a 19 + 2 format, synthesised with two 3' deoxythymidines (dT) overhangs. An unmodified non-self-delivery (non-sd-) lamin A/C siRNA (Lamin A/C non-sd-siRNA; sequence: 5'-CUGGACUCCAGAAGAACA) targeting human lamin A/C mRNA was designed and synthesised by Eurofins MWG Operons (Ebersberg, Germany). A nonspecific unmodified green fluorescent protein (GFP) siRNA (Control non-sd-siRNA; sequence: 5'-GGCUACGUCCAGGAGCGCACC) targeting the GFP mRNA not present in the human keratinocytes model was used as a control.

Accell modified self-delivery (sd-) CD44 siRNA (Accell CD44 sd-siRNA) and non-Accell modified non-sd-siRNA (siSTABLE CD44 non-sd-siRNA) targeting human CD44 mRNA (both siRNA sequence: 5'-GGCGCAGAUCGAUUUGAAU) [33] were designed and synthesised by Dharmacon Products, Thermo Fisher Scientific (Lafayette, CO, USA). A nonspecific self-delivery K6a_513a.12 siRNA (Accell control sd-siRNA) targeting a keratin 6a mutation not present in the human keratinocytes model or the mouse skin model was used as control [34].

2.1.2 *In vivo* studies—Accell modified sd-siRNA targeting the CBL coding region of transgenic hMGFP/CBL mouse mRNA (5'-UAACGAUCCACGACGUAAA; Accell CBL3 sd-siRNA) were designed and synthesised by Dharmacon Products, Thermo Fisher Scientific (Lafayette, CO, USA). Accell control sd-siRNA was used as control.

2.2. Microneedle fabrication and coating

2.2.1. Microneedle fabrication, coating and characterisation—Stainless steel microneedle devices (containing either 5 or 10 needles of 700 μm length and 200 μm base width) were fabricated from a stainless steel sheet by wire electrical discharge machining (EDM). A coating method was developed to coat siRNA onto the surface of the microneedles from a liquid formulation (Figure 1A).

To determine the efficiency and reproducibility of the coating method, 3 μL of unmodified siRNA (Dharmacon Products, Thermo Fisher Scientific, Lafayette, CO, USA) (70 mg mL^{-1} in phosphate buffered saline; PBS) was loaded into a 20 μL pipette tip as a reservoir for coating. Microneedles (6 devices with 5 microneedles per array) were coated with siRNA using the method described in detail in Figure 1A and were allowed to dry at 4°C for either 1 or 20 hours (h) (3 devices for each drying time) to provide a *theoretical* loading of 35 μg siRNA coated onto each microneedle device. The method was repeated with another 6 devices with 10 microneedles per array. To determine *actual* loading, siRNA was recovered from the microneedle devices by washing in 150 μL siRNA buffer for 5 minutes (min) and the nucleic acid concentration was quantified using the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

To visually characterise siRNA coating onto microneedles, 1 μL of BLOCK-iT™ Alexa 647 fluorescent siRNA (1 mg mL^{-1} in PBS) (a gift from Dr. Xavier de Mollerat du Jeu, Life Technologies, USA) was loaded into a pipette tip and microneedles (10 microneedles per array) were coated with siRNA using the method described in Figure 1A to provide a theoretical loading of 1 μg siRNA onto the microneedle device. The coated microneedles

were imaged using the Leica DM IRB epifluorescence microscope and imaging system. Dry coated microneedles were manually inserted into excised human breast skin, obtained from surgical procedures with informed consent and full ethical approval, left seated for 10 min and then removed. The siRNA fluorescence remaining on the microneedles post-delivery was imaged.

2.2.2 Microneedle preparation for *in vitro* studies—Initial *in vitro* studies compared naked siRNA against siRNA complexed with a lipid-based transfection reagent (Lipofectamine™ RNAiMAX; Invitrogen, Life Technologies, UK), termed a “lipoplex.” Using the coating method described in Figure 1A, naked lamin A/C non-sd-siRNA pre-coating formulation (48 pmol in 8 μ L PBS) or lamin A/C non-sd-siRNA lipoplex pre-coating formulation (48 pmol in 8 μ L Lipofectamine™ RNAiMAX) was coated onto steel microneedle devices (4 devices per treatment group; each with 5 microneedles per array) to provide a *theoretical* loading of 12 pmol siRNA on each microneedle device. Coated microneedles were allowed to dry at 4°C for 1 h before the siRNA was recovered by washing microneedles in 50 μ L siRNA buffer for 5 min. The recovered siRNA solutions were used to treat cells as described in section 2.3.2.

Accell control or Accell CD44 sd-siRNA was coated onto microneedle devices to determine the effect of microneedle coating on the stability of sd-siRNA. A *theoretical* loading of 1.5 nmol siRNA was coated onto each microneedle device, which were subsequently allowed to dry at 4°C for 10 h before the siRNA was recovered by washing the microneedles in 60 μ L siRNA buffer for 5 min. The recovered siRNA solutions (20 μ L of recovered siRNA solution containing approximately 500 pmol siRNA) were used to treat cells as described in section 2.3.2.

2.2.3. Microneedle preparation for *in vivo* studies—Two μ L of siRNA coating solution (70 mg mL⁻¹ for Accell sd-CBL3 and 80 mg mL⁻¹ for Accell sd-Control) in PBS was loaded into a pipette tip reservoir for coating. The steel microneedle devices (4 devices per treatment group; each with 10 microneedles per array) were coated with siRNA using the coating method described in Figure 1A to provide a *theoretical* loading of 35 μ g and 40 μ g Accell CBL3 and Accell Control sd-siRNA coated onto each microneedle device, respectively. Coated microneedles were maintained at 4°C for up to 18 h before use.

2.3 *In vitro* siRNA stability studies

2.3.1 HaCaT cell culture—Immortalised human keratinocyte cells (HaCaT cells) [35] were received as a gift from Dr. Mark Gumbleton (School of Pharmacy and Pharmaceutical Sciences, Cardiff University). Cells were cultured in a growth medium consisting of Dulbecco's modified Eagle medium (DMEM), supplemented with 10 % foetal bovine serum (FBS), 100 unit mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin (all Life Technologies, UK) at 37°C in a humidified atmosphere containing 5% CO₂.

2.3.2 Cell treatment—Before treatment, cells were seeded into 12-well plates at a density of 2.5×10^4 cells cm⁻² in 1 mL growth medium and maintained for 24 h. Cell populations were then treated using (i) GFP non-sd-siRNA 10 nM lipoplex (non-targeting control), (ii) naked lamin A/C non-sd-siRNA 10 nM (non-lipoplex control), (iii) lamin A/C non-sd-siRNA lipoplex 10 nM (positive control), (iv) naked lamin A/C non-sd-siRNA pre-coating formulation diluted to 10 nM formed into lipoplex (naked siRNA pre-coat + lipo) (v) naked lamin A/C non-sd-siRNA recovered from microneedles and subsequently formed into lipoplex (naked siRNA coated + lipo), (vi) lamin A/C non-sd-siRNA lipoplex pre-coating formulation diluted to 10 nM (siRNA lipoplex pre-coat) (vii) lamin A/C non-sd-siRNA lipoplex formulation recovered from microneedles (siRNA lipoplex coated) and (viii) Opti-

MEM[®] solution (untreated). The final concentration of lipoplexes in treatment groups with lipoplex was 1% v/v Lipofectamine[™] RNAiMAX transfection reagent. Transfection complexes prepared according to the supplier's recommended protocol were diluted with Opti-MEM[®] solution (Life Technologies, UK) to a volume of 200 μ L and added to the seeded cells to a final transfection volume of 1200 μ L. The final concentration of siRNA across all treatment groups with siRNA was 10nM (12 pmol in 1200 μ L transfection volume). Cells were treated in quadruplicate transfection with triplicate sample for mRNA quantification by RT-qPCR and the remaining treatment sample for protein analysis by western blotting. Treated cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

For the Accell sd-siRNA study, cells were seeded into 24-well plates at a density of 2.5×10^4 cells cm⁻² in 0.5 mL growth medium and maintained for 24 h. Cell populations were then treated using (i) Accell control sd-siRNA 1 μ M (Accell non-targeting control), (ii) siSTABLE CD44 non-sd-siRNA 1 μ M (non-Accell CD44 control), (iii) Accell CD44 sd-siRNA 1 μ M (Accell CD44 positive control), (iv) Accell CD44 sd-siRNA recovered from microneedles 1 μ M (Accell CD44 coated). HaCaT cells were treated with the siRNA diluted in serum-free Accell delivery media (ThermoFisher Scientific, UK) at a volume of 500 μ L according to the supplier's recommended protocol. HaCaT cells were treated by replacing the seeding media with the delivery mixture containing siRNA. Due to the passive self-delivery nature of the Accell siRNA, a higher final siRNA concentration of 1 μ M (500 pmol in 500 μ L treatment volume) is required for efficient gene silencing *in vitro*. Treated cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h, the treatment media was replaced with DMEM supplemented with 10% FBS and the cells were incubated for a further 24 h. Cells were treated in triplicate transfection for mRNA quantification by RT-qPCR.

2.3.3 mRNA quantification—Forty-eight hours following treatment, total RNAs from lamin A/C non-sd-siRNA and Accell CD44 sd-siRNA treated cells were isolated using the Ambion[®] PureLink[™] RNA Mini Kit (Life Technologies, UK), quantified (NanoVue spectrophotometer; GE Healthcare, UK) and stored at -80°C. Reverse transcription converted total RNA into first-strand cDNA using random primers with the High Capacity cDNA Reverse Transcription system (Applied Biosystems, Life Technologies, UK). Quantitative PCR was performed using the ABI 7900HT Fast Real-Time PCR system with TaqMan[®] Fast Advanced Master Mix (Applied Biosystems). Target gene inhibition was measured using Taqman gene expression assays specific for lamin A/C (Hs00153462_m1) or CD44 (Hs00153304_m1) and the endogenous control GAPDH (Hs02758991_g1). All data points reported are the mean and standard error of three separate treatments each with three replicate qPCR assays.

2.3.4 Protein analysis—Following the transfection period (48 h), cells treated with lamin A/C non-sd-siRNA were washed and incubated with ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 1% Triton X-100) containing protease inhibitor cocktail (Roche, UK). The lysates were incubated on ice for 10 min prior to centrifugation at 13 000 g at 4°C for 10 min. Protein content was determined using a BCA (bicinchoninic acid) assay (Sigma Aldrich, UK). Samples containing 15 μ g protein were mixed with SDS sample buffer, heated at 95°C for 5 min before being separated by electrophoresis on 10% resolving Mini-PROTEAN TGX precast gels (Bio-Rad, UK) using the Bio-Rad Mini Protean 3 system (Bio-Rad, UK). Proteins were transferred to nitrocellulose papers (Bio-Rad, UK) using the Trans-Blot[®] Turbo Transfer System[™] (Bio-Rad, UK), probed with primary antibodies recognising lamin A/C (ab8984) (Abcam, UK) and α -tubulin (T9026) (Sigma Aldrich, UK) and detected using a HRP conjugated secondary antibody (32430) (Thermo Fisher

Scientific, UK) and Super Signal West DURA solutions (Thermo Fisher Scientific, UK) developed onto Amersham Hyperfilm ECL (GE Healthcare, UK).

2.4 *In vivo* studies

2.4.1 Animal models—Tg CBL/hMGFP mice [29] were bred at Stanford University, Stanford, California, USA. Tg CBL/hMGFP mice were crossed with *skh1* hairless mice, purchased from Charles River (Wilmington, MA), to constitute the transgenic hairless reporter mouse Tg-h CBL/hMGFP. Mice were screened for reporter expression by intraperitoneal injection of luciferin (100 μ l of 30 mg/ml; 150 mg/kg body weight) and the live anesthetized (2% isoflurane) mice were imaged 10 min later using the IVIS 200 Imaging System (Caliper Life Sciences). All animals were treated according to the guidelines of both the National Institutes of Health and Stanford University.

2.4.2 Mouse paw treatment—Mice were anaesthetised with 2-3% isoflurane prior to microneedle administration. A group of four Tg-h CBL/hMGFP mice were treated on the right paw with microneedles (10 microneedles of 700 μ m length) coated with Accell CBL3 sd-siRNA. The counterpart left paw was treated with identical microneedles loaded with Accell non-targeting control sd-siRNA. The microneedle devices were manually inserted into the middle region of the mouse paw (between the footpads) and held in place for 5 min. Devices remained in the skin for an additional 15 min (total insertion time 20 min) before being removed. Treatments were repeated at the same location daily for 10 days, except day 2. At day 10, the mice were sacrificed and the treated paw skin was removed by surgical dissection.

2.4.3 Quantification of siRNA deposition—The mass of siRNA loaded onto each microneedle device, prior to application, was estimated from the mass of siRNA in the known volume of coating formulation that was used to coat the microneedles. Following application, the microneedles were rinsed using a fixed volume of buffer (100 or 150 μ L) for 5 min and the nucleic acid concentration was quantified using the NanoDrop spectrophotometer. The mass of nucleic acid deposited into the mouse skin during each treatment procedure was inferred by the mass balance between the loaded siRNA and the siRNA remaining on the microneedles after removal from mouse paws. Unpaired two-tailed t-tests were performed to determine the statistical difference of siRNA deposited in the control and CBL3 group.

2.4.4 Intravital fluorescence imaging and quantification—Prior to treatment, mice were anaesthetised with 2-3% isoflurane and imaged intravitaly using the Maestro Optical imaging system (Caliper LifeSciences now part of Perkin Elmer, USA), as previously described [30, 36]. Imaging was repeated on days 2, 4, 6, 8 and 10 of the treatment regimen. Each spectrum was set by unmixing the autofluorescence from a hairless *skh1* negative non-hMGFP expressing mouse from the spectra of a Tg-h CBL/hMGFP positive mouse analysed in parallel. The conditions and subject positioning for image acquisition was standardized, facilitating meaningful comparison of data. Quantitative data was extracted using ImageJ software (National Institute of Health, USA) by selecting the treatment area and calculating the average signal (counts $s^{-1} mm^{-1}$) at the various time points. The ratio of average signal in the right (treatment) versus left (control) paws was determined in each mouse and was normalised to the pre-treatment levels.

2.4.5 Skin sectioning and immunofluorescence—Skin tissue from the region between the footpads of one mouse was excised, embedded in OCT and frozen on dry ice. Ten μ m sections (Leica CM3050S Cryostat; Leica Microsystems (UK) Ltd, UK) were captured on microscope slides and mounted with VECTASHIELD® Mounting Medium

containing $1.5 \mu\text{g mL}^{-1}$ 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd., UK) for nuclear staining. Transgene fluorescence was visualised using the Leica DM IRB epifluorescence microscope and imaging system (Leica Microsystems Ltd, UK).

2.4.6 Gene expression quantification—RNA was isolated from the skin tissue (obtained from three mice per cohort), reverse transcribed and subjected to qPCR as previously described [29]. A Taqman gene expression assay, specifically designed for hMGFP was used (hMGFP-F: 50-CCCCAAGGACATCCCTGACT; hMGFP-R: TGCTTCGCTCCCACGAGTA and probe: 6FAM-TCAAGCAGACCTTCCCCGA-MGBN FQ; Applied Biosystem, USA). TaqMan gene expression assay specific for CD44 (Hs00153304_m1) was used as the endogenous gene control. All data points reported are the mean and standard error of three replicate assays.

2.5 Data processing and statistical analysis

Graphs were generated and statistical analyses (unpaired two-tailed t-tests) performed using Prism[®] 5 for Mac OS X (GraphPad Software Inc. USA).

3. Results

3.1 Efficiency and reproducibility of siRNA coating onto microneedles

The pipette coating method, as shown in Figure 1A, was used to coat stainless steel microneedles with siRNA. Two sets of microneedle devices of identical dimensions (Figure 1B) but with different density of microneedles per array (5 or 10 microneedles) were coated. The same volume and concentration of siRNA solution was used to coat each set of arrays. Figure 2 shows that the pipette reservoir method for microneedle coating resulted in a meaningful and relatively reproducible (approximately $35 \mu\text{g}$) mass of siRNA coated onto and recovered from each array of microneedles. The recovery of siRNA was not affected by prolonged (20 h) post-drying of the microneedles.

3.2 siRNA distribution on microneedles before and after skin insertion

To determine the extent of microneedle coating and subsequent *in situ* release of the siRNA, stainless steel microneedles were coated with a fluorescently-tagged siRNA, allowed to dry, imaged, applied to excised human skin and then re-imaged (Figure 3). Figure 3A shows a relatively uniform coating of siRNA on the surface of a single representative steel microneedle. Following a 10 min insertion into skin, the vast majority of the coating was removed from the microneedle surface (Figure 3B).

3.2 Functionality of siRNA recovered from coated microneedles

In vitro cell studies were performed to determine whether siRNAs retain biologically activity following coating on steel microneedles. Lamin A/C gene expression in HaCaT keratinocyte cells was analyzed at both the mRNA (qPCR) (Figure 4A) and protein (western blotting) levels (Figure 4B). Lamin A/C mRNA levels were significantly ($P < 0.0001$) reduced in cells treated with siRNA that had been previously coated onto microneedles, recovered and subsequently complexed with Lipofectamine[™] RNAiMAX (naked siRNA + lipo) prior to treatment. The level of siRNA-mediated lamin A/C reduction (85.4%) was comparable to that achieved with the positive control (non-coated siRNA lipoplex 10nM; 85.2% reduction). When siRNA was pre-complexed with the transfection reagent prior to coating and recovery from microneedles (siRNA lipoplex coated) there was no reduction in mRNA synthesis. Lamin A/C protein expression studies were also performed to determine whether reduction in mRNA levels correlated to a reduction in protein expression. Figure 4B confirms that the reduction in mRNA following treatment with lamin A/C siRNA, that has

been coated and recovered from microneedles and subsequently complexed with transfection reagent (naked siRNA coated + lipo), translates to reduced lamin A/C protein expression in HaCaT cells. siRNA pre-complexed with a transfection reagent (siRNA lipoplex) prior to coating and recovery from microneedles (siRNA lipoplex coated) did not confer a reduction in protein expression.

Coating microneedles with a binary lipoplex appears to compromise the *in vitro* activity of the siRNA, which is likely to translate to limited gene silencing efficiency *in vivo*. Given the encouraging stability of naked siRNA coated onto microneedles, a further *in vitro* stability study was performed using modified “self-delivery” naked siRNA constructs. Accell sd-siRNAs enter cells passively without the need for lipid-based transfection reagents and are also modified for improved stability. CD44 mRNA expression in HaCaT keratinocyte cells treated with the modified sd-siRNA was analyzed by RT-qPCR (Figure 5). CD44 mRNA levels were significantly ($P < 0.0001$) reduced in cells treated with both the sd-siRNA that had been previously coated onto microneedles and recovered (Accell CD44 coated; 67.4% reduction) and the Accell positive control (Accell CD44 sd-siRNA 1 μ M; 74.5% reduction). Naked CD44 non-sd-siRNA did not reduce mRNA synthesis when applied to the cells in the absence of a transfection reagent (non-Accell CD44 control).

3.3 Delivery of microneedle coated sd-siRNA and gene silencing in transgenic mouse skin

Informed by the results of the *in vitro* siRNA stability studies, subsequent experiments examined the ability of Accell-modified sd-siRNA coated stainless steel microneedles (Figure 2) to facilitate gene silencing *in vivo*. The central region of the CBL/hMGFP mouse paws were treated with Accell CBL3 sd-siRNA targeted against the CBL coding region of the hMGFP/CBL mRNA. Treatments were conducted daily and the formulation was delivered as a dry coat. The doses of Accell control and CBL3 sd-siRNA were approximately 40 μ g and 35 μ g per microneedle device, respectively. The mass of siRNA deposited into the skin was relatively low (less than 15 μ g) on day 1 (Figure 6A). Thereafter, approximately 50% to 85% of loaded siRNA (in the range of 20-30 μ g) was deposited in the mouse paws during each treatment. The dose of control siRNA that was deposited on day 1, 5 and 9 was significantly ($p < 0.05$) higher than CBL3 siRNA but the delivery of functional siRNA was never significantly higher than the control siRNA.

At day 10, mice were sacrificed and the treated skin area was harvested from three mice for RT-qPCR analysis (Figure 6B). The level of hMGFP mRNA was reduced in two out of three mice, with mouse 2 and mouse 3 showing a mean relative hMGFP mRNA reduction of 49% and 38%, respectively. No gene silencing was detectable at the mRNA level in Mouse 1.

Intravital fluorescence images were captured every other day throughout the treatment regime (Figure 6C provides examples). The images captured using the Maestro imaging system were compared to determine the effect of siRNA treatment on hMGFP protein expression (Figure 6D). The fluorescence signal intensity at the sites treated with microneedles coated with CBL3 siRNA showed a reduction in hMGFP protein expression after 8 and 10 days as compared to the paws administered with the non-targeting control siRNA. The reduction in signal intensity at day 10 ranged from 35% to 50%.

Paw skin from one mouse was also harvested and analysed using established histology methods to characterise the degree and depth of change in fluorescence signal intensity (Figure 6E). Skin sections treated with the non-targeting control (upper panels of Figure 6E) exhibit the intense GFP signal arising from the transgenic protein in the upper layers of the epidermis [29]. In the CBL3 treated paw (lower panels of Figure 6E) the fluorescent signal is clearly reduced. These images, taken from two separate treatment areas, are representative

of all the transverse sections of the analysed samples. Brightfield images (Figure 6E) indicate that the stratum corneum is intact in the regions of low fluorescence, confirming that the depleted fluorescence is due to a reduction in protein rather than physical disruption of the skin.

4. Discussion

Greater understanding of the RNAi pathway is allowing researchers to study and modify gene function in an unprecedented way [4]. However, the lack of suitable and effective delivery tools for siRNA is a major barrier to clinical exploitation. For example, whilst siRNA has shown promise as an effective corrective therapy in severe genetic dermatological conditions, the conventional hypodermic needle and syringe delivery method was intolerable to patients [22]. Microneedles are able to penetrate the stratum corneum barrier of human skin in a minimally invasive manner [37, 38] to enable effective nucleic acid delivery [31, 32, 39-44]. The use of steel microneedles to deliver molecules, macromolecules and vaccines, as a dried coating, is now well established [28, 31, 32, 45, 46]. However, this system has not previously been tested for the delivery of siRNA to skin.

In order for a coated microneedle delivery system to be effective, several factors need to be considered, including efficient and stable microneedle coating formulations and procedures, effective skin penetration performance and targeted and efficient drug deposition [28, 45, 47]. A drug formulation with sufficient viscosity and surface tension is important for uniform distribution of coated materials on the microneedle surface. A coating formulation that is sufficiently water-soluble is also important to enable the drug to dissolve quickly upon insertion of the microneedles into the skin [32]. Moreover, the mechanical strength and adhesive properties of a dried coating should be sufficient to retain contact with the microneedles during their insertion into skin [28]. siRNA is a small double-stranded RNA molecule that is highly water-soluble. Therefore, a simple aqueous formulation of fluorescently labelled siRNA was used to coat the surface of steel microneedles. The coating procedure, involving placing a formulation-loaded pipette tip over the microneedles, withdrawing, allowing 30 seconds for drying and then re-applying, led to a relatively reproducible and uniform coating of the microneedles. Using this method, we were able to load up to 40 µg siRNA onto microneedles, which is an order of magnitude higher than the nanogram quantities of siRNA that have been loaded onto dissolvable microneedles in previous studies [30, 33]. Following insertion into human skin and microscopic inspection of the microneedles, the coating dissolved leaving only residual fluorescence on the needle surface.

Having established a simple formulation and method to reproducibly coat appropriate quantities of siRNA onto steel microneedles, we further investigated the functional stability of the coated siRNA. Non-viral nucleic acid delivery commonly involves the use of lipid-based cationic transfection reagents to facilitate cell uptake and processing. The commercially available transfection reagent Lipofectamine™ RNAiMAX proved to be effective in transfecting the immortalised human keratinocyte cell line employed in this study, with no significant effect on cell toxicity (data not shown). This transfection reagent is also effective in transfecting cultured monolayer primary human keratinocyte cells isolated from excised human breast skin tissue (data not shown) and could potentially therefore be useful for *ex vivo* or *in vivo* applications.

When siRNA was coated onto microneedles, allowed to dry and recovered, the siRNA remained fully functional as evidenced by marked reduction of lamin A/C mRNA level and protein expression of lamin A/C in cells treated with siRNA. In contrast to this, the biological functionality of siRNA, when pre-complexed with Lipofectamine™ RNAiMAX

diminished upon the microneedle coating, drying and recovery processes. We speculate that the coating and drying processes could result in a change in the structural conformation of the electrostatic lipoplex complex and/or compromised changes to the lipid reagent. It is known that nucleic acid-liposome complexes can form aggregates upon storage, resulting in reduced transfection efficiency and necessitating preparation of complexes immediately before administration [48]. A number of studies have however demonstrated the ability to freeze-dry, freeze-thaw or spray-dry siRNA-liposome and DNA-liposome complexes in the presence of sugars as lyoprotectants, with minimal effect on lipoplex functionality [48, 49]. Indeed, as carbohydrate-enriched formulations have shown to improve the physical stability of nucleic acid, in this case plasmid DNA, upon microneedle coating [32, 46], the value of these stability-enhancing formulations when coating siRNA onto steel microneedles is worthy of investigation in future studies.

Accell-modified “self-delivery” siRNA does not require a transfection agent to facilitate cell transfection. *In vitro* functionality studies revealed that following the coating and recovery process Accell sd-siRNA remained functional, as demonstrated by a significant reduction of CD44 mRNA expression in HaCaT cells treated with Accell sd-siRNA targeting the CD44 gene. Furthermore, as previous studies have shown gene silencing in skin treated with Accell sd-siRNA coated onto dissolvable microneedles [30, 33] this form of sd-siRNA was used to assess the *in vivo* functionality of siRNA delivered via coated steel microneedles in a transgenic mouse model. The coating procedure described in this study was able to dry coat up to 40 µg of Accell sd-siRNA per array of 10 microneedles. Recovery and quantification of siRNA from microneedles following insertion into mouse model skin suggests that 50% to 85% of the coated siRNA was deposited in the mouse paw. The reduced deposition observed on treatment Day 1 was likely attributable to inexperience in microneedle application in this model, leading to inadequate skin insertion. The delivery efficiency of nucleic acids from coated microneedles is a function of the coating formulation and its distribution on the needle, the depth of microneedle penetration into skin and the hydration status of the tissue proximal to the microneedles [32]. In this instance the major limitation is likely to be needle insertion depth, as it proved technically challenging to fully insert microneedles into the contoured footpad area. Moreover, the manual coating procedure inevitably results in some of the material being coated on the base of the microneedle device (Figure 3). This material would not be deposited in the skin but would be quantifiable post-insertion. Nevertheless, both the actual dose and percentage deposited in the treatment area were significantly greater than previously reported, where an estimated dose of 120 ng siRNA (10% of coated dose) was administered using biodegradable microneedle arrays [30]. The greater utilization of coated material, with less wastage, could have important cost and efficacy implications when delivering expensive biological therapeutics using microneedles.

The transgenic mouse model used in the *in vivo* studies expresses GFP in the upper epidermis (granular layer and stratum corneum), enabling functional assessment of intradermally administered siRNA [29]. Visualisation and quantification of protein production, through fluorescence intensity, was used to determine gene silencing. Quantification of fluorescence intensity in the CBL3-treated mouse paw (but not the non-specific control-treated paw) indicates that microneedle delivery of siRNA resulted in a discernible reduction in protein expression. The observed reduction in reporter protein signal was confirmed by fluorescence microscopy of skin sections, which illustrated a clear reduction in GFP signal in the upper regions of the epidermis, where the hMGFP protein is predominantly expressed in this model. Transverse sections confirmed that the reduction in fluorescence was not an artefact, i.e. it was attributable to reduced protein expression rather physical disruption to the epidermis caused by the microneedle treatment. To determine siRNA gene silencing at the mRNA level, RT-qPCR quantification of hMGFP mRNA was

performed from total RNA isolated from mice at the end of the 10-day treatment. The supportive, yet equivocal, nature of this mRNA data (a clear reduction of mRNA was seen in two out of the three mice tested) reflects the degree of challenge of inducing and analysing gene silencing *in vivo*. In *in vitro* studies, dividing cell monolayers can be exposed directly to high concentrations of siRNA and those cells can be easily recovered and characterised for gene expression. Accurately and reproducibly delivering siRNA to a three-dimensional tissue architecture and evaluating the functionality of the nucleic acid at a sub-cellular level within that tissue is a far greater challenge. Nevertheless, taken together, the protein and mRNA data presented in this study suggest that the siRNA coated onto, and released from, steel microneedles remains functional and can be effectively delivered to skin to facilitate localised gene silencing *in vivo*.

A major limiting factor for microneedle systems is the dosing capacity. Gonzalez-Gonzalez and colleagues have previously shown that delivery of Accell sd-siRNA from biodegradable microneedles can induce gene silencing in the paws of a similar transgenic CBL/hMGFP reporter mouse [30]. However, the loading capacity of these devices is restricted and thus dry-coated solid steel microneedles may provide an alternative for enhanced loading capacity [31, 32]. The enhanced loading capacity of the steel microneedles did not translate to gene silencing efficiency above that observed in a previous microneedle study where an estimated 120 ng of siRNA was administered [30]. However, in the previous study, siRNA was delivered over a wider area using three separate arrays of 4 × 5 microneedles (60 in total) at each time-point compared to the single row of 10 microneedles that was employed in this study. It is possible that fewer microneedle penetrations could restrict the cell numbers exposed to the siRNA treatment, thus leading to a compromised gene silencing efficiency. It is also apparent however that publications targeting siRNAs to skin rarely observe a silencing effect in excess of 50% [29, 30, 33, 50], regardless of siRNA dose or delivery method, and therefore a further reduction in gene expression may not be possible, even at greater delivered dose. Whilst the higher loading capacity achieved in our study lead to no obvious advantage in mouse skin over the biodegradable arrays used in this previous study, given the simplicity and improved loading capacity of steel microneedles, this may well be advantageous in human skin tissue in conditions where a larger dose of therapeutic siRNA needs to be delivered.

These results serve to demonstrate, for the first time, the ability to deliver siRNA using coated solid microneedles, resulting in reporter protein silencing *in vivo*. siRNA delivery using steel microneedles is attractive as such devices are simple and cost-effective for large-scale manufacture. Once coating formulations and processes are further optimised and automated, this system could provide a practical minimally invasive, patient-friendly, self-administration alternative for the delivery of therapeutic nucleic acids to the skin. Given these encouraging data the next stage is to determine the effectiveness of siRNA delivery using microneedles in human skin.

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Figure 1.

(A) Simple and precise microneedle coating method. An illustration of siRNA coating onto the surface of steel microneedles. (i) A volume of siRNA of known concentration was loaded into a pipette tip as a reservoir for coating. (ii) Microneedles were coated with siRNA by repeated immersions of the needles into the reservoir, with 30 seconds of air-drying time between each immersion. (iii) Microneedles were coated until the reservoir was exhausted. (iv) The coated microneedles were allowed to further dry at 4°C. **(B) Microneedle morphology.** An array of ten steel microneedles of 700 μm length. The inset micrograph image shows the microneedle geometry in greater detail. (Bar=500 μm).

Figure 2. siRNA coating onto steel microneedles

A theoretical mass of 35 μg of siRNA per microneedle device was loaded onto two sets of microneedle devices with different density of microneedles per array (6 with 5 microneedles per array and 6 with 10 microneedles per array). siRNA from 3 devices was recovered from each set of microneedle devices at the drying time-points. (h = hour; n = 3; error bar = standard deviation)

Figure 3. Extent of siRNA coating pre and post skin insertion

Fluorescence images of a microneedle coated with approximately 0.1 μg of fluorescent siRNA pre- (**A**) and post- (**B**) insertion into excised human breast skin. The images shown are representative of other microneedles analysed in the array. (Bar=200 μm).

Figure 4. Retention of siRNA functionality following microneedle coating. Lamin A/C mRNA (A) and protein (B) expression was determined in HaCaT cells 48 h post-treatment with siRNA (A) Lamin A/C mRNA expressions were relative to the non-targeting negative control group and normalised to GAPDH endogenous control gene levels. (n = 3 transfection repeats, each with 3 qPCR assay replicates; ***significant reduction in mRNA expression compared with negative control, $p < 0.0001$). (B) Lamin A/C protein expression with α -tubulin as the protein loading control. Lamin A/C siRNA recovered from microneedle devices (naked siRNA coated) was subsequently complexed with 1% v/v LipofectamineTM RNAiMAX for transfection in HaCaT cells. The same amount of siRNA (10nM) was used across all the treatment groups. (Non-targeting control = GFP siRNA 10nM + 1% v/v LipofectamineTM RNAiMAX; non-lipoplex control = lamin A/C siRNA 10nM; positive control = lamin A/C siRNA 10 nM + 1% v/v LipofectamineTM RNAiMAX; naked siRNA pre-coat + lipo = lamin A/C siRNA lipoplex pre-coating formulation diluted to 10nM + 1% v/v LipofectamineTM RNAiMAX; siRNA lipoplex pre-coat = lamin A/C siRNA pre-coating formulation diluted to 10nM siRNA with 1% v/v LipofectamineTM RNAiMAX; siRNA lipoplex coated = lamin A/C siRNA lipoplex formulation (10nM siRNA + 1% v/v LipofectamineTM RNAiMAX) recovered after coating onto microneedles).

Figure 5. Retention of Accell sd-siRNA functionality following microneedle coating. CD44 expression was determined in HaCaT cells 48 h post-treatment with siRNA

CD44 mRNA expressions were relative to the Accell non-targeting negative control group and normalised to GAPDH endogenous control gene levels. (n = 3 transfection repeats, each with 3 qPCR assay replicates; ***significant reduction in mRNA expression compared with Accell non-targeting control, $p < 0.0001$). (Accell non-targeting control = Accell control sd-siRNA 1 μM ; non-Accell CD44 control = siSTABLE CD44 non-sd-siRNA 1 μM ; Accell CD44 positive control = Accell CD44 sd-siRNA 1 μM ; Accell CD44 coated = Accell CD44 sd-siRNA recovered after coating onto microneedles, 1 μM).

Figure 6. hMGFP reporter gene and protein expression in transgenic CBL/hMGFP mouse paws microneedle treated with Accell CBL3 or control sd-siRNA over a 10-day treatment regime (A) Mass of siRNA deposited in mouse paws from the dry-coated microneedle devices. siRNA deposition in skin was estimated by subtracting the quantity of siRNA remaining on the microneedle devices (10 microneedles) from the theoretical loaded quantity on treatment days 1, 4, 5, 7 and 9 of the 10-day regime. *Denotes a statistically significant ($p < 0.05$; unpaired two-tailed t-test) difference between control and treatment groups. (B) RT-qPCR quantification of hMGFP mRNA expression. hMGFP mRNA levels from the CBL3 treated paw were quantified relative to the non-targeting control treated paw and normalised to CD44 endogenous gene control. Each column corresponds to the mean of three qPCR replicates and the error bars indicate standard error. (C) hMGFP expression in the paws of Mouse 2 (as a representative example), imaged intravitaly with the Maestro imaging system. Fluorescence is pseudocoloured green. (D) **Quantification of hMGFP protein expression.** Intravital images captured using the Maestro imaging system were processed through ImageJ and quantified. The ratio of the average signal of the CBL3 treated area and non-targeting control treated area is normalised to day 0 (pre-treatment). (E) Fluorescence microscopy of cross-sections (10 µm) of treated mouse paws following 10 days of treatment. hMGFP protein expression (or lack thereof) was visualised by fluorescence microscopy of samples from mouse paws treated with Accell non-targeting control (top panel) or CBL3 (bottom panel) sd-siRNA. Two sets of representative images are shown from different areas of the skin samples. In each set, the left panel shows the fluorescence overlay and the right panel shows the corresponding bright-field images. hMGFP fluorescence is pseudocoloured green. Nuclei were stained with DAPI and pseudocoloured blue. (Bar=100µm).