

| Title                          | Hydrodynamic gene delivery in human skin using a hollow<br>microneedle device   |
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| Authors                        | Dul, M.;Stefanidou, M.;Porta, P.;Serve, J.;O'Mahony,<br>Conor;Malissen, B.;Henri, S.;Levin, Y.;Kochba, E.;Wong, F.<br>S.;Dayan, C.;Coulman, S. A.;Birchall, J. C.   |
| Publication date               | 2017-02-28  |
| Original Citation              | Dul, M., Stefanidou, M., Porta, P., Serve, J., O'Mahony, C.,<br>Malissen, B., Henri, S., Levin, Y., Kochba, E., Wong, F. S., Dayan,<br>C., Coulman, S. A. and Birchall, J. C. (2017) 'Hydrodynamic gene<br>delivery in human skin using a hollow microneedle device',<br>Journal of Controlled Release. doi:10.1016/j.jconrel.2017.02.028 |
| Type of publication            | Article (peer-reviewed)   |
| Link to publisher's<br>version | 10.1016/j.jconrel.2017.02.028   |
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| Download date                  | 2025-07-31 23:13:29   |
| Item downloaded<br>from        | https://hdl.handle.net/10468/4919   |



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### Accepted Manuscript

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| PII:           | S0168-3659(17)30087-1              |
|----------------|------------------------------------|
| DOI:           | doi: 10.1016/j.jconrel.2017.02.028 |
| Reference:     | COREL 8678                         |
| To appear in:  | Journal of Controlled Release      |
| Received date: | 3 January 2017                     |
| Revised date:  | 24 February 2017                   |
| Accepted date: | 25 February 2017                   |
|                |                                    |

Please cite this article as: M Dul, M Stefanidou, P Porta, J Serve, C O'Mahony, B Malissen, S Henri, Y Levin, E Kochba, FS Wong, C Dayan, SA Coulman, JC Birchall , Hydrodynamic gene delivery in human skin using a hollow microneedle device. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Corel(2017), doi: 10.1016/j.jconrel.2017.02.028

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# Hydrodynamic gene delivery in human skin using a hollow microneedle device

Dul M<sup>1</sup>, Stefanidou M<sup>1</sup>, Porta P<sup>1</sup>, Serve J<sup>1</sup>, O'Mahony C<sup>4</sup>, Malissen B<sup>5</sup>, Henri S<sup>5</sup>, Levin Y<sup>3</sup>, Kochba E<sup>3</sup>, Wong FS<sup>2</sup>, Dayan C<sup>2</sup>, Coulman SA<sup>1,\*</sup> and Birchall JC<sup>1</sup>

\*Corresponding author

1 Cardiff School of Pharmacy and Pharmaceutical Sciences, Cardiff University,

Cathays Park, Cardiff, CF10 3NB, UK

2 School of Medicine, Cardiff University, Cardiff, UK.

3 NanoPass Technologies, Israel

4 Tyndall National Institute, Lee Maltings, University College Cork, Cork, Ireland

5 Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, Inserm,

CNRS, Marseille, France

#### Abstract

Microneedle devices have been proposed as a minimally invasive delivery system for the intradermal administration of nucleic acids, both plasmid DNA (pDNA) and siRNA, to treat localised disease or provide vaccination. Different microneedle types and application methods have been investigated in the laboratory, but limited and irreproducible levels of gene expression have proven to be significant challenges to pre-clinical to clinical progression. This study is the first to explore the potential of a hollow microneedle device for the delivery and subsequent expression of pDNA in human skin. The regulatory approved MicronJet600® (MicronJet hereafter) device was used to deliver reporter plasmids (pCMVß and pEGFP-N1) into viable excised human skin. Exogenous gene expression was subsequently detected at multiple locations that were distant from the injection site but within the confines of the bleb created by the intradermal bolus. The observed levels of gene expression in the tissue are at least comparable to that achieved by the most invasive microneedle application methods e.g. lateral application of a microneedle. Gene expression was predominantly located in the epidermis, although also evident in the papillary dermis. Optical coherence tomography permitted real time visualisation of the sub-surface skin architecture and, unlike a conventional intradermal injection, MicronJet administration of a 50µl bolus appears to create multiple superficial microdisruptions in the papillary dermis and epidermis. These were co-localised with expression of the pCMV $\beta$  reporter plasmid. We have therefore shown, for the first time, that a hollow microneedle device can facilitate efficient and reproducible gene expression of exogenous naked pDNA in human skin using volumes that are considered to be standard for intradermal administration, and postulate a hydrodynamic effect as the mechanism of gene delivery.

**Keywords:** Microneedles, human skin, DNA, genetic vaccine, gene therapy, hydrodynamic

#### 1. Introduction

Intradermal delivery of plasmid DNA (pDNA) has been proposed for a number of therapeutic applications (Abdul-Wahab et al. 2014, Hart and Vile, 1994), including DNA vaccines, which are targeted to the skin to exploit the highly evolved and competent local immune system (Robinson, 1999, Kutzler and Weiner, 2008). However, although detectable expression of exogenous genes in human and animal skin was achieved over 20 years ago (Hengge et al. 1995, Hengge et al. 1996, Hengge et al. 1998, Yu et al. 1999), clinical translation has been hindered by extra-and intra-cellular barriers to efficient and reproducible cell transfection.

Cell barriers to transfection have been well characterised in numerous cell types, including skin cells. Viral vectors enable efficient and long-term expression of exogenous genes (Howarth et al. 2010) but they have a number of acknowleded constraints (Boulaiz et al. 2005, Lv et al. 2006, Mingozzi and High 2013, Thomas et al. 2003). Non-viral vectors provide an alternative, but whilst these vectors are comparably safe, not immunogenic, low cost and easy to manufacture (Li and Huang, 2000, Wang et al. 2013), their transfection efficiency is often significantly reduced compared to their viral counterparts (He et al. 2010, Nayerossadat et al. 2012). Physical enhancement techniques have also been used promote cell transfection. These include electroporation (Broderick et al. 2011, Drabick et al. 2001, Guo et al. 2011, Roos et al. 2009), iontophoresis (Brand and Iversen, 1996, Vlassov et al. 1996), particle bombardment (Klein et al. 1987, Yang et al. 1990), tattooing (Bins et al. 2005, Oosterhuis et al. 2012), ultrasound (Newman and Bettinger, 2007) and radiofrequency ablation (Birchall et al. 2006). However these approaches are limited by the complexity and cost of the equipment, cell death and restricted access to the tissue or organ of interest (Meacham et al. 2014). The challenge of gene delivery at the cellular level is compounded in the skin, where a diverse range of cell populations exist in a complex three dimensional architecture. The most attractive target for localised gene expression in this stratified organ is the region with the greatest density of viable proliferating cells, i.e. the basal layer of the viable epidermis. This target layer exists 100 - 200 µm below the skin surface and is sandwiched by the terminally differentiating keratinocytes of the upper epidermis and the underlying connective tissue of the dermis. Potential targets for nucleic acid therapies also exist in the elastin and collagen packed layer of the dermis but cells in this region are not constrained to a membranous organisation and are often motile. Overcoming the physical and biological barriers of the skin, which has evolved to act

as a barrier to exogenous materials and insults, to target exogenous nucleic acids to specific cell types is therefore challenging.

Despite these challenges, numerous studies have demonstrated that vectorless (naked) pDNA can transfect cells in both human and animal skin (Coulman et al. 2006, Hengge et al. 1995, Hengee et al. 1996, Yan et al. 2014). This has been traditionally achieved via conventional intradermal injection, whereby a single hypodermic needle with millimetre dimensions is used to overcome the stratum corneum barrier and transfer a bolus of pDNA into the skin. However the resulting gene expression levels are low and often restricted to the viable epidermal cells that surround the injection site (Hengge et al. 1996). This relatively inefficient method of delivery has therefore been augmented by physical transfection methods, such as electroporation (Broderick et al. 2011, Wells, 2004), which temporarily permeabilises cell membranes to enhance transfection levels in the tissue. Recent advances in this area, including Phase 2 clinical trial data (Trimble et al. 2015), are very encouraging. However conventional injections combined with electroporation have acknowleged limitations e.g. pain, bleeding and the inherent costs and complexity associated with electroporation technology. The ideal delivery system would therefore be a single and simple minimally invasive device (Miller and Pisani, 1999) that can achieve vectorless delivery of nucleic acids across both the tissue barrier, the stratum corneum, and the major cellular barrier, the cell membrane (lbraheem et al. 2014).

Microneedle (MN) technology uses single or multiple microscopic needles to deliver therapeutic materials through the stratum corneum (SC) barrier into the underlying viable epidermis, or dermis, in a minimally invasive manner. The primary advantage of MNs, compared to other 'physical' delivery methods, is the simplicity of the device. Studies have exemplified the potential of MNs for delivery of nucleic acids, both pDNA and siRNA, for the treatment of genetic skin diseases, cutaneous cancers, hyperproliferative diseases and vaccinations and these are summarised in a recent review (McCaffrey et al. 2015). These studies have employed solid MNs to deliver pDNA into the skin using either the "soak and poke" (Birchall et al. 2005, Coulman et al. 2006, Pearton et al. 2008, Yan et al. 2014) or the "coat and poke" approach (DeMuth et al. 2013, Gill et al. 2010, González-González et al. 2011, Kim et al. 2013, Pearton et al. 2012). The former involves topical application of a liquid pDNA formulation prior to application of a MN device (Pearton et al. 2008). The "soak and poke" method results in reasonable levels of gene expression (Birchall et al. 2005, Chabri et al. 2004, Coulman et al. 2006, Pearton et al. 2008, Yan et al. 2005, Chabri et al. 2004, Coulman et al. 2006, Pearton et al. 2008, Yan et al.

2014) however it is impossible to control the delivered dose using this method. The alternative strategy is to "coat and poke" i.e. a MN device is dry coated with a pDNA formulation and then applied to the skin (DeMuth et al. 2013, González-González et al. 2011, Pearton et al. 2012). MN coating methods are able to control the spatial distribution and mass of a therapeutic that is loaded onto MNs and thus dosing is more reproducible for the coat and poke approach (Kim et al. 2013). However the transfection efficiency of this method is significantly reduced (Pearton et al. 2012). Both of these approaches suffer from low to moderate levels of gene expression that are restricted to cells adjacent to the site of microdisruption. Transfection can be heightened by increasing tissue disruption using repeated MN applications to the skin surface (Yan et al. 2014) or lateral application of MNs (Mikszta et al. 2002). However, these approaches cause significant disruption of the tissue barrier that are unlikley to be clinically acceptable.

The hollow MN approach administers the therapeutic cargo in a liquid formulation through one or more needles of micron dimensions. The advantages of hollow MN systems are conferred by its ability to inject a finite dose using a standard syringe and with minimal training. Re-formulation of commercially available medicines and vaccines is often not required and therefore the potential of hollow MNs has been established both pre-clinically and clinically (Daugimont et al. 2010, Gupta et al. 2009, Gupta et al. 2012, Kim and Prausnitz, 2011, Levin et al. 2014, Levin et al. 2015, Norman et al. 2013, Roxhed et al. 2008, Van Damme et al. 2009, Wang et al. 2006). To the best of our knowledge, the ability of hollow MNs to deliver nucleic acids to human skin has not yet been reported. The aim of this study was to investigate the potential of a regulatory approved hollow MN device, MicronJet (Levin et al. 2015), for pDNA delivery to human skin in an effort to combine the favourable transfection efficiencies previously witnessed with liquid delivery (soak and poke approach) with the finite dosing that can be achieved using a hollow MN delivery system.

#### 2. Materials and methods

All reagents and laboratory consumables were purchased from Fisher Scientific, UK, (Loughborough, UK), unless otherwise indicated.

2.1 Ethics statement

Human skin samples were obtained from female patients undergoing breast reduction surgery or mastectomy under informed written patient consent and local ethical committee approval (South East Wales Research Ethics Committees Panel C, Reference: 08/WSE03/55).

#### 2.2 MN characterization

The hollow MN devices (MicronJet) used in this study was provided by NanoPass Technologies Ltd. The device consists of an array of three MNs, each 600 µm in length, bonded to the tip of plastic adapter, which could be mounted on a standard hypodermic syringe. The needles were fabricated from silicon using MEMS (Micro Electro Mechanical Systems) technology (Van Damme et al. 2009). MicronJet MNs were visualised using a Stemi 2000-C Stereomicroscope (Zeiss, Welwyn Garden City, UK).

#### 2.3 Preparation of pDNA

The reporter plasmids pCMVβ and pEGFP-N1 were amplified and purified from previously transformed stocks of DH5α *Escherichia coli* using antibiotic selective conditions and a Qiagen Mega Kit (Qiagen Ltd, Crawley, UK), (Birchall et al. 2005). The pIRES-TdTomato plasmid was obtained from Clontech. Plasmid propagation and purification was performed using a Qiagen Mega Kit (pCMVβ and pEGFP-N1) or Qiagen HiSpeed Midi Kit (pIRES-TdTomato plasmid) (Qiagen Ltd, Crawley, UK).

2.4 Collection, processing and maintenance of an ex vivo human skin

Excised human breast skin was collected immediately after surgery and transported to the laboratory in culture media, which consisted of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 100 unit mL<sup>-1</sup> of penicillin and 100  $\mu$ g mL<sup>-1</sup> of streptomycin (all Life Technologies, UK) at 4°C. Collected skin samples were used within 2 h of excision. Subcutaneous adipose tissue was removed by blunt dissection and skin was pinned, dermis side down, onto a cork dissection board to facilitate injection. An 8 mm biopsy punch was used to isolate areas of treated skin, which were further maintained in the organ culture by incubating at the air-liquid interface at 37°C in an atmosphere of 5% (v/v) CO<sub>2</sub>/95% (v/v) air (Ng et al. 2009). Data in this study was obtained from replicate samples obtained from ten independent skin donors (aged 51- 80 years old).

2.5 Gene expression in primary and continuous human keratinocyte cell lines

2.5.1 HaCaT cell culture

Immortalised human keratinocyte cells (HaCaT cells), (Boukamp et al. 1988) were received as a gift from Professor Mark Gumbleton (School of Pharmacy and Pharmaceutical Sciences, Cardiff University). Cells were cultured in a growth medium, which consisted of DMEM, supplemented with 10% fetal bovine serum (FBS), 100 unit mL<sup>-1</sup> of penicillin and 100  $\mu$ g mL<sup>-1</sup> of streptomycin (all Life Technologies, UK) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

#### 2.5.2 Primary keratinocytes isolation and culture

Superficial skin tissue was mechanically isolated from a full thickness skin sample using a surgical blade (approx.  $1x1 \text{ cm}^2$ ) and transferred to a 0.25% trypsin solution which was incubated overnight at 4°C. The resulting suspension of cells was filtered through a 70 µm cell strainer and centrifuged at 300 x *g* for 7 mins. The cell pellet was gently re-suspended in media (EpiLife medium supplemented with 10% human keratinocyte growth supplement (HGKS) and 100 unit mL<sup>-1</sup> penicillin, 100 µg mL<sup>-1</sup> of streptomycin and 2.5 µg mL<sup>-1</sup> of amphotericin B (all Life Technologies, UK) before seeding in a 25cm<sup>2</sup> flask. Growth media was changed every 2-3 days until the primary keratinocytes reached 70-80% confluence.

#### 2.5.3 HaCaT cell transfection

Twenty four hours before treatment, cells were seeded into 24-well plates at a density of  $7.5x10^4$  cells cm<sup>-2</sup> in 0.5 mL growth media. Cell populations were then treated using either (i) naked plasmid (pEGFP-N1 or pIRES-TdTomato) or (ii) pDNA lipoplex formed with Lipofectamine2000 transfection reagent (positive control). Naked plasmid (0.6 µg in 100 µL PBS) was added to a well containing 500 µL of DMEM. Transfection complexes were prepared according to the manufacturer's protocol (Life Technologies, UK). Briefly, 2 µL of Lipofectamine2000 was diluted in 50 µL of DMEM and pDNA (0.6 µg /per well) was diluted in 50 µL of DMEM. After 5 min of incubation, pDNA and Lipofectamine2000 dilutions were mixed gently and allowed to equilibrate for 20 min at room temperature. Cells were washed once with PBS, and then lipoplexes (100 µL) were added to each well containing 500 µL of DMEM and mixed gently by rocking the plate back and forth. After 6 h, the cells were washed three times with PBS and the cells were incubated for a further 42 h. All experiments were performed at least in triplicate.

#### 2.5.4 Primary keratinocyte cell transfection

Primary keratinocyte transfection was performed in a comparable manner to HaCaT transfection (Section 2.5.3), with the following exceptions. Twenty four hours before

treatment cells were seeded into 24-well plates at a density of 5x10<sup>4</sup> cells cm<sup>-2</sup> in 0.5 mL growth media. pDNA lipoplexes were prepared in EpiLife media and cells were treated as detailed in 2.5.3. After 6 h the treatment was removed and the cells were washed three times with PBS. EpiLife media, supplemented with 10% HGKS, was then added to the cell populations which were incubated for a further 42 h. All experiments were performed at least in triplicate.

#### 2.5.5 Examining gene expression in vitro

Following a 42 h incubation period (Section 2.5.3 and 2.5.4), cell populations were washed three times with PBS before addition of EpiLife imaging media (Life Technologies, UK) to permit live cell imaging. The presence of GFP and TdTomato in cell monolayers was visualised using the Leica DM IRB epifluorescence microscope and imaging system (Leica Microsystems Ltd, UK). The transfection efficiency of pEGFP-N1 and pIRES-TdTomato was determined by flow cytometry using the FACSVerse flow cytometry system (BD Biosciences, UK). To prepare for FACS analysis cells were washed with PBS and then treated with trypsin-EDTA at 37°C for 10 min. The cell suspension was then re-suspended in growth medium, centrifuged (Thermo Fisher Scientific, UK) at 300 × g for 5 min and washed with PBS. Pelleted cells were fixed with 100 µL of fixation buffer (BD Biosciences, UK) for 15 min at 4°C, washed and re-suspended in 200 µL of staining buffer (BSA), (BD Biosciences, UK) before flow cytometry analysis. GFP fluorescence was detected using a 527/32 filter. while TdTomato used a 568/42 filter (PE channel). On each sample, a minimum of 10,000 events was collected. Analysis of collected data was performed with the FlowJo Flow Cytometry Analysis Software for Mac Version 10. (Tree Star Inc., USA). The percentage of gated cells in each sample indicates the percentage of fluorescent cells.

#### 2.6 MN mediated delivery of pDNA in vitro

Twenty four hours before transfection HaCaT cells were seeded into 24-well plates at density of  $7.5 \times 10^5$  cells cm<sup>-2</sup> in 0.5 mL growth media. Cell populations were then treated using: (i) naked plasmid (pEGFP-N1; 5 µg per well) injected into the media by pipette, (ii) naked plasmid (pEGFP-N1; 5 µg per well) injected into the media by MNs and (iii) pDNA lipoplex (pEGFP-N1; 5 µg per well) formed with Lipofectamine2000 transfection reagent (prepared according supplier's recommended protocol) as a positive control. Transfection was performed using a comparable protocol to that described in Section 2.5.3. The transfection efficiency of

pEGFP-N1 in cells was determined by fluorescent microscopy and flow cytometry (Section 2.5.5).

#### 2.7 Gene expression in ex vivo human skin

#### 2.7.1 Conventional and MN injection of pDNA into human skin

Following collection and preparation of excised skin (Section 2.4) 50  $\mu$ L of pDNA (pCMV $\beta$  and pEGFP-N1) diluted in PBS, at a range of concentrations (detailed in results), was injected into the tissue using MicronJet MNs or a traditional hypodermic needle (BD Microlance 3, 26G x 3/8"). 50  $\mu$ L of PBS was injected as a negative control. Following MN injection, the treatment sites were excised using 8 mm biopsy punches and incubated for 24 h at the air-liquid interface at 37°C and 5% (v/v) CO<sub>2</sub>/95% (v/v) air. Injections were performed at least in triplicate.

#### 2.7.2 Lateral MN disruption of human skin

Split-thickness human skin was pinned onto a cork dissection board (Section 2.4). 20  $\mu$ l of pDNA solution (pCMV $\beta$ ) at concentration of 3.5 mg/ml was applied to the skin surface. A 4x4 array of MN-like structures, manufactured using silicon wet etching techniques (Wilke et al. 2005), was provided by the Tyndall National Institute. In this instance the wafer was removed from the etchant early in order to leave a flat uppermost surface, Figure 8D (insert), instead of the nm-sharp tip associated with conventional microneedles (O'Mahony, 2014). The flat tip of this truncated conical structure (more accurately termed a frustum tip) helps to enhance the skin disruption and maximise reliability when scraped laterally across the skin surface. This array was then applied in a downward motion to the edge of the treated area, under constant pressure, for a period of approximately 2 secs and subsequently moved laterally across the skin surface.

#### 2.7.3 Examination of plasmid expression in ex vivo human skin

#### 2.7.3.1 β-galactosidase enzyme detection

Twenty four hours after treatment, all samples were rinsed in PBS for 30 min, and fixed in 2% glutaraldehyde/PBS at 4°C for 2 h. Subsequently, each sample was rinsed three times in PBS/ 4 mM MgCl<sub>2</sub> for 2 h. Samples were then immersed in X-Gal staining solution (0.2% X-Gal, 2 mM MgCl<sub>2</sub>, 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub> prepared in PBS) for up to 16 h at 37°C. Following three times 30 minute PBS rinses the skin sample was visualised *en face* using a Stemi 2000-C Stereomicroscope (Zeiss, Welwyn Garden City, UK) and a KL1500 electronic external light source (Schott UK Limited, Stafford, UK), prior to each sample being embedded in OCT

medium and stored at -80°C.

#### 2.7.3.2 Preparation of skin cryosections and H&E staining

Skin samples embedded in OCT blocks were sectioned using a Cryostat FSE (Thermo Scientific Shandon, UK). Skin sections (10  $\mu$ m) were collected onto Superfrost Plus microscope slides and observed with an Olympus BX50 microscope (Olympus, Middlesex, UK). Selected slides were subsequently stained using haematoxylin and eosin (H&E) to visualize the skin architecture (Fischer et al. 2008).

#### 2.7.3.3 Intravital fluorescence imaging

Skin treated with pEGFP-N1 plasmid was incubated for 24 h at 37°C, rinsed in PBS and fixed in 4% paraformaldehyde/PBS at 4°C for 24 h. Skin samples were then rinsed in PBS and imaged intravitally using the Kodak FX-Pro imaging system (Bruker Corporation), with wavelengths set to 470 nm (excitation) and 535 nm (emission).

#### 2.7.3.4 Fluorescence microscopy of skin sections

Following 24 h incubation, skin samples were rinsed in PBS and fixed in 4% paraformaldehyde/PBS at 4°C for 24 h. Skin was then cryosectioned (2.7.3.2) and imaged using the Leica DM IRB epifluorescence microscope and imaging system (Leica Microsystems Ltd., UK).

#### 2.7.3.5 Preparation of epidermal sheets and fluorescent microscopy

Skin treated with pEGFP-N1 was incubated for 24 h at 37°C before epidermal sheets were isolated by heat separation. Skin biopsy punches were incubated in water at 60°C for 55 seconds after which the epidermis was removed from the dermis using forceps under a dissection microscope. Isolated epidermal sheets were then washed in PBS, fixed in acetone for 20 min at -20°C, washed again and mounted on slides using Dako fluorescent mounting medium (Dako, UK). The tissue was then visualised by a Leica DMI6000B confocal microscope (Leica Microsystems (UK) Ltd, UK) with LAS AF software (Leica Microsystems (UK) Ltd, UK).

2.7.3.6 Fluorescence analysis of skin cells by flow cytometry

Excised skin was maintained in organ culture for 24 h prior to removal of the lower dermis by blunt dissection (Ng et al. 2009). The remaining tissue was immersed in an enzyme solution containing Dispase II (2 units/ml) (Roche, UK), Collagenase I (197units/ml) and Deoxyribonuclease I (DNA-se I) from bovine pancreas (20 units/ml) in RPMI 1640 (all Invitrogen, UK) for 1 h at 37°C. Epidermal sheets were

then mechanically separated from the dermis using forceps and placed in 0.25% (w/v) solution of trypsin for 30 min at 37°C. Following incubation, the epidermal sheets were pipetted up and down to increase cell yield and the suspension of the cells was passed through a 70  $\mu$ m cell strainer. Single cell suspensions were subsequently centrifuged at 400 x g for 10 min and washed in PBS. Pelleted cells were fixed with 100  $\mu$ L of fixation buffer (BD Biosciences, UK) for 15 min at 4°C, washed and re-suspended in 200  $\mu$ L of stain buffer (BSA), (BD Biosciences, UK) before flow cytometry analysis (Section 2.5.5).

2.8 Optical coherence tomography (OCT) of human skin following MN delivery

Excised human skin was removed from -20°C storage and allowed to equilibrate to room temperature for approximately 2 h. Sub-cutaneous adipose tissue was removed by blunt dissection and skin was pinned, dermis side down, onto a cork dissection board to facilitate injection. Real time injections of methylene blue were visualised using the VivoSight System (Michelson Diagnostics, UK). The system is a Swept Source Fourier Domain OCT with clinical CE and FDA approval. This MultiBeam OCT system with <7.5 µm lateral resolution and <5 µm axial resolution in tissue is capable of achieving between 1.2 and 2.0 mm imaging depth and a 6×6 mm lateral field of view. Each OCT scan consists of 60 B-scans (in the x–y plane) with 4 µm pixels, separated by 100 µm in the y direction.

#### 2.9 Data processing

Image processing including scale bar inclusion was performed ImageJ software (National Institute of Health, USA). Statistical analysis of differences between transfection was determined by t-test analysis using GraphPad Prism software version 6.0c.

#### 3. Results

#### 3.1 Characterisation of MN devices

MicronJet is a CE marked and FDA cleared device consisting of three MNs, each 600  $\mu$ m in length with a lumen that is approximately 60  $\mu$ m in diameter, bonded to a plastic adapter that permits attachment to a Luer fit syringe (Fig 1).



Fig. 1.

A hypodermic needle with intradermal-bevel (26-gauge) and Nanopass MicronJet 600µm needles (brightfield microscopy).

#### 3.2 In vitro transfection of immortalised and primary keratinocytes

Immortalised keratinocytes (HaCaT) were used to exemplify the functionality of the pEGFP-N1 and pIRES-TdTomato reporter plasmids. Freshly isolated human keratinocytes were subsequently used to determine transfection efficiency as these cells are more representative of the *in vivo* environment. A significant proportion (44.1  $\pm$  0.2%) of HaCaT cells were positive for gene expression following treatment with the pEGFP-N1:Lipofectamine2000 lipoplex (Fig 2A). The detection of gene expression was reduced (10.1  $\pm$  0.4%) in cells transfected with pIRES-TdTomato:Lipofectamine2000 (Fig 2D). Gene expression efficiency in freshly isolated primary skin cells was reduced by an order of magnitude, with 3.8  $\pm$  0.4% of the cell population transfected by pEGFP-N1 lipoplexes, and 0.8  $\pm$  0.1% for pIRES-TdTomato lipoplexes. Positive transfection by GFP and TdTomato was confirmed by fluorescence micrographs of HaCaT cells (Figure 2B and 2E) and primary keratinocytes (Figure 2C and 2F).



#### Fig. 2.

Transfection efficiency of (A) pEGFP-N1 and (D) pIRES-TdTomato plasmid, as determined by flow cytometry of immortalised and primary human keratinocyte cell lines. Representative fluorescence micrographs of HaCaT (B, E) and primary keratinocyte human cell lines (C, F) treated with pEGFP-N1 (B, C) and pIRES-TdTomato (E,F) lipoplexes with Lipofectamine2000 are also included to provide qualitative analysis. pDNA dose 0.6  $\mu$ g in each treatment group. Statistical analysis: \* p<0.05, \*\*\*\* p<0.0001.

#### 3.3 Evaluating MicronJet mediated delivery of plasmid DNA in human skin

Experiments in viable human skin explants were performed to determine gene expression efficiency *ex vivo* following plasmid delivery via MicronJet MNs. Figure 3 provides representative *en face* images of X-gal stained *ex vivo* human skin, 24 h after MicronJet delivery of the pCMV $\beta$  reporter plasmid (Figure 3B-3D and 3F-3H) or PBS as a control (Figure 3A and 3E). Histological sections indicate that MN penetration of human skin typically creates disruptions in human skin to a depth of 200-250 µm and a width of 150 µm (Figure 3A). Gene expression, visualised as areas of punctate intense blue staining, is extensive with multiple sites of reporter protein staining (Figure 3B and 3C). Successful transfection was evident in at least three independent experiments in different skin donors and at all pCMV $\beta$  doses delivered (22.5 µg, 50 µg and 250 µg). *En face* images and transverse sections (Figure 3B, 3C, 3F and 3G) suggest that gene expression occurs predominantly in the viable epidermis although gene expression can also been observed in the dermis

(Figure 3D, 3G and 3H). It is interesting to note that the site of gene expression was remote from the injection site (Figure 3B and 3C). Furthermore, whilst injection of naked plasmid DNA resulted in positive gene expression, injection of pCMV $\beta$  plasmid (22.5 µg) complexed with Lipofectamine 2000 did not result in detectable gene expression in *ex vivo* human skin (data not shown).



#### Fig. 3.

Images of ex vivo human skin following MicronJet 600µm injection of (A) PBS as a negative control or (B-H) naked pCMVß plasmid (250 µg in 50 µl), followed by incubation in X-gal solution. (A, B, C) provide en face images of the skin surface and (D) is the underside of a skin sample i.e. viewed from the dermis. Transverse cryosections (10µm) illustrate the (E) microneedle insertion site and successful transfection of pCMVß plasmid in the (F) epidermis, (G) epidermis and dermis and (H) dermis of the skin. Areas of transfection are highlighted by arrows. Donor skin in these exemplar images was obtained from 71 (A-C, E-H) and 54 years old females.

Intravital imaging of human skin explants 24 h after MicronJet injection of naked pEGFP-N1 plasmid (300  $\mu$ g) confirmed the results observed with pCMV $\beta$ , with the expressed GFP causing a significant increase in the fluorescence intensity for all four treated skin samples compared to the negative control. The pattern of localised expression also supports previous observations with pCMV $\beta$ , i.e. there is evidence of increased fluorescence distant from the injection site but within the confines of the raised bleb that is produced upon MicronJet injection of 50 $\mu$ l of pDNA solution (Figure 4B). Fluorescence imaging of epidermal sheets isolated from skin samples post-transfection validates the positive gene expression witnessed in intravital



images (Figure 4C and 4D), whilst traditional histology methods confirm that gene expression occurs predominantly within the viable epidermis (Figure 4E and 4F).

#### Fig. 4.

Reporter gene expression in human skin tissue following MicronJet 600µm injection of naked pEGFP-N1. (A, B) Intravital images of four 8mm human skin biospy punches following injection of (A) PBS or (B) naked pEGFP-N1 plasmid (300 µg) using the MicronJet 600µm. Representative fluorescence micrographs showing positive gene expression in (C, D) human epidermal sheets treated with (C) 300 µg or (D) 50 µg of naked pEGFP-N1 plasmid. (E, F) are 10 µm cryosections of human skin following microneedle injection of 300 µg naked pEGFP-N1. Donor skin was obtained from 75 (A-B, E-F) and 60 years old females.

In a further experiment *ex vivo* human skin was treated with pEGFP-N1 delivered via MicronJet MNs 24 hours prior to epidermal cells being extracted from the tissue and analysed by fluorescence activated cell sorting (FACS). FACS plots indicate that a small population of the extracted epidermal cells were GFP positive (Figure 5B). Positive expression of the reporter protein in these skin-derived cells is further supported by fluorescence microscopy (Figure 5D).



#### Fig. 5.

Detectable GFP in epidermal cells that were isolated from freshly excised human skin following MicronJet 600 µm injection of naked pEGFP-N1 (250 µg). Control skin samples received an injection of PBS. Flow cytometry plots of (A) control epidermal cells and (B) epidermal cells transfected with pEGFP-N1 plasmid. Fluorescence micrographs of (C) a control epidermal cell suspension and (D) positive GFP expression within the epidermal cell suspension that has been isolated from human skin post-treatment. Donor skin was obtained from a 67 years old female.

3.4 Understanding MicronJet-mediated delivery of plasmid DNA at the cellular level

To determine the influence of the delivery method on transfection, pEGFP-N1 was delivered to a HaCaT cell monolayer either using a variable volume pipette or using

MicronJet MNs. Lipoplexes of pEGFP-N1 with Lipofectamine2000 (positive control) facilitated transfection of a significant proportion of cells (Fig 6B and 6D). Flow cytometry and fluorescence micrographs indicate that naked pEGFP-N1, delivered via MicronJet MNs (Figure 6C and 6E), can successfully transfect a small proportion of cells *in vitro*. However, this low level of gene expression was not observed when naked pEGFP-N1 was applied to the cell monolayer using a pipette (Figure 6A).



#### Fig. 6.

Representative flow cytometry plots and accompanying micrographs to illustrate pEGFP-N1 expression in HaCaT cells following treatment of the cell monolayer by different delivery strategies. Gene delivery was facilitated by (A) topical naked plasmid (5 µg) delivery from a pipette, (B, D) topical lipoplex (pEGFP-N1 plasmid with Lipofectamine2000) delivery from a pipette (positive control) or (C, E) naked plasmid (5 µg) extruded on to the cell monolayer through a MicronJet 600µm.

#### 3.5 Understanding MicronJet mediated delivery of plasmid DNA at the tissue level

Optical coherence tomography (OCT) captured real time changes in the skin architecture during bleb formation by the MicronJet device. Localised disruption of the tissue at the point of injection was accompanied by adjacent distinct minor tissue disruptions, exclusive to the area of bleb formation (see Suppl. Figure 1A-C and Figure 7B and 7C). These microdisruptions appeared in a region of the tissue that is approximately 200-500µm below the skin surface and are a feature that were not

witnessed in untreated skin (Figure 7A) or during conventional intradermal injections (see Suppl. Figure 1A). Subsequent histological examination of the tissue following injection of pCMV $\beta$  supported OCT images, showing in some cases positive gene expression localised to areas of significant tissue disruption (Figure 7D, 7E and 7F).



#### Fig. 7.

(A, B,C) OCT images highlight (A) untreated and (B) microneedle treated ex vivo human skin, both (B) during and (C) after a 50µl microneedle injection of methylene blue using MicronJet 600µm needles. In (B) the microneedle can be identified adjacent to the bleb that is being created. The dashed orange line highlights the

border between the epidermal and dermal layers and the white dashed line in (C) indicates the edge of the bleb created by the microneedle injection. Arrows are used to highlight disruptions in the human skin architecture at positions that are distant from the microneedle insertion point, but within the confines of the bleb. Naked pCMV $\beta$  (250 µg in 50µl) was also injected into ex vivo human skin using the MicronJet 600µm and (D, E, F) light microscopy of 10 µm cryosections highlights pCMV $\beta$  expression proximal to mechanical disruptions (highlighted by a dashed blue line) in the skin tissue. Donor skin was obtained from 70 (A, B), 69 (C) and 71 (D,E,F) year old females.

Conventional intradermal injection of pCMV $\beta$  into *ex vivo* human skin resulted in gene expression in cells of the viable epidermis that are frequently adjacent to the point of injection (Figure 8B and 8C). Histological evaluation of the skin indicated that transfection is either associated with the epidermal cells that are proximal to the point of needle entry into the skin (the needle track, Figure 8E) or, if the tip of the needle was positioned in a cellular region, at the tip of the needle (both of these features were identified in a single sample in Figure 8F). Transfection following conventional intradermal injection of commensurate volumes of pCMV $\beta$  was therefore less extensive and significantly reduced compared to that observed with MicronJet injection.



#### Fig. 8.

En face images of ex vivo human skin treated with naked pCMVß plasmid and stained with X-gal solution. Skin samples were intradermally injected using a hypodermic needle to deliver (A) PBS (negative control), (B) 250µg of pCMVß in 50µl

or (C) 20µg of pCMVß in 20 µl into ex vivo skin tissue. (D) Alternatively the skin was treated by lateral application of a silicon microneedle array inset) following pretreatment of the skin with 70µg of a pCMV $\beta$  solution in 20µl. (E,F,G,H) Cryosections illustrate the location of transfection in the stratified tissue following (E, F) intradermal injection or (G,H) lateral application of the silicon microneedle array. Donor skin was obtained from 71 (A, B), 51 (C, E, F) and 76 (D, G, H) year old females.

To further explore the role of mechanical tissue disruption in mediating localised gene expression, silicon MN-like frustums were used to scarify the skin surface following topical liquid application of the pCMV $\beta$  plasmid. Lateral movement of the frustums across the skin surface resulted in multiple discrete areas of gene expression proximal to the mechanical microdisruptions (Figure 8D). Transverse sections confirmed plasmid expression in the viable epidermis and frustum induced mechanical microdisruptions in the upper layers of the tissue i.e. the epidermis and the papillary dermis (Figure 8G and 8H).

#### 4. Discussion

There is a growing body of research that describes delivery of pDNA to murine (Gill et al. 2010, González-González et al. 2011, Kumar et al. 2012, Kim et al. 2014, Song et al. 2012) and other animal (DeMuth et al. 2013, Yan et al. 2014) models using solid MNs, particularly in the context of vaccination. Whilst these studies provide valuable information related to biological end-points and also exemplify the potential of solid MNs for pDNA delivery, the gross architecture and biology of animal and human skin have acknowledged differences, including the density, locations, proportions and phenotypes of both epidermal and dermal cells. Studies have also evaluated pDNA delivery in a viable *ex vivo* human skin model (Ng et al. 2009), but whilst studies have demonstrated proof-of-concept (Birchall et al. 2005, Coulman et al. 2006, Daugimont et al. 2010, McCaffrey et al. 2015, Pearton et al. 2012, Song et al. 2012), the 'soak and poke' and 'coat and poke' strategies are associated with limitations related to dosing reproducibility and transfection efficiency.

In this study hollow MNs (Figure 1) were used, for the first time, for the minimally invasive delivery of a pDNA solution into human skin. Experiments using two different reporter plasmids indicate that following MicroJet delivery of a known bolus dose of pDNA into human skin, transfection is more extensive and reproducible (Figure 3, 4) than that previously observed using the "soak and poke" (Birchall et al. 2005, Coulman et al. 2006, Pearton et al. 2008, Yan et al. 2014) or "coat and poke" MN approaches (DeMuth et al. 2013, Gill et al. 2010, González-González et al. 2011, Kim et al. 2013, Pearton et al. 2012, Song et al. 2012). Most notably, the punctate areas of intense gene expression are remote from the injection site. This suggests that the MicronJet device facilitates transfection by a different mechanism than other published MN delivery strategies. A series of experiments were therefore conducted to elucidate the mechanism by which gene expression is enhanced upon MicronJet delivery of reporter pDNA to *ex vivo* human skin.

Initial studies used a well-characterised immortalised human keratinocyte cell line (HaCaT cells) to exemplify the functionality of the reporter pDNA. Cell delivery using a commercial non-viral transfection reagent produced transfection rates of over 40% (Figure 2), however this did not translate to primary keratinocytes (Figure 2). The nuclear envelope is the major intracellular barrier to nuclear delivery of pDNA (Dean et al. 2005) and therefore transfection efficiencies are greatest in rapidly dividing cells, where the nuclear envelope is briefly but repeatedly compromised during mitosis (Escriou et al. 2001, Mortimer et al. 1999, Nicolau and Sene 1982, Wilke et al. 1996). Primary cells proliferate slowly and therefore transfection efficiencies in these cells are reduced. In terminally differentiating primary keratinocytes, where nuclear entry relies on passive translocation via the nuclear pore complex (Mattaj and Englmeier, 1998, Stoffler et al. 1999, Wilson et al. 1999), this problem is exacerbated. Therefore whilst immortalised keratinocytes and non-viral vectors are useful laboratory tools, the more representative primary keratinocyte cells used in this study exemplify the cellular barriers to transfection that exist *in vivo*.

Treatment of the skin with a solid MN temporarily disrupts the stratum corneum and the plasma membranes of underlying viable cells. It has therefore been hypothesised that pre-treatment of the skin surface with a topical pDNA solution prior to application of a MN device ("soak and poke") can facilitate almost simultaneous passage of pDNA across the skin barrier and into the cytoplasm of the cells in the viable epidermis (Pearton et al. 2012, Yan et al. 2014). If the cell membrane recovers after

this mechanical trauma, and cell viability is maintained, intracellular delivery of pDNA will translate to expression of the exogenous gene product. The drawback of this mechanism of delivery is the exclusive proximity of positively-expressing cells to the MN insertions, which necessitates extensive or repeated MN applications to facilitate reasonable levels of gene expression. "Coat and poke" (Pearton et al. 2012) and "poke and soak" (Chabri et al. 2004, Mikszta et al. 2002, Pearton et al. 2008, Yan et al. 2014) strategies produce lower levels of gene expression due to the time delay between MN induced disruption of the cell membranes and exposure of the compromised cells to the pDNA. This delay is dependent on either the dissolution rate of the relatively insoluble macromolecular nucleic acid within the local environment of the skin tissue ("coat and poke") or the interval between treatment of the skin with the MN device and topical application of a pDNA solution ("poke and soak"). Therefore a significant observable enhancement in both the level and distribution of gene expression following the use of hollow MNs, compared to their solid counterparts, suggests that this method of MN delivery improves cellular delivery as well as providing more reproducible and controlled dosing into the tissue.

Delivery of a liquid bolus into human skin using a hollow MN may be considered to be more comparable to a conventional intradermal injection than solid MNs. However, the level and location of gene expression observed in ex vivo human skin following delivery of pDNA by hollow MNs is also significantly greater than that achieved using conventional intradermal injection (Figure 8B). Conventional intradermal injections use a 260µm needle bore and expel the liquid bolus in a single stream, compared to Micronjet, which uses three 60µm bores. The smaller bore sizes of the MicronJet increase the exit velocity of the liquid when a comparable downward pressure is applied to the syringe. The increased velocity is likely to facilitate greater direct cell transfection. In vitro experiments partially exemplified this point (Figure 6), with increased transfection evident when the naked pDNA was applied to cell monolayers using the MicronJet device compared to a pipette tip, although absolute gene expression levels remain low. Direct cell transfection by a high velocity liquid is also supported by a significant body of literature related to jet injections (Cartier et al. 2000, Fargnoli et al. 2014, Fargnoli et al. 2016, Furth et al. 1995, Sawamura et al. 1999, Walther et al. 2001, Walther et al. 2002). However, although a jet injection mechanism may contribute to the heightened gene expression observed in this study, the limited number of jets and the extensive distribution of gene expression suggest that it is unlikely to be the sole or major reason for the enhancement.

pDNA delivery using hollow MNs results in gene expression in cells remote from the point of injection, but which are restricted to the 'bleb' that is created by the intradermal liquid bolus (Figures 3 & 4). This 'bleb', as it is commonly referred to, is a discrete area of skin tissue that temporarily expands in response to the intradermal administration of a liquid volume. This type of event is also witnessed during the hydrodynamic delivery of nucleic acids to animal models (Liu et al. 1999, Zhang et al. 1999, Bonamassa et al. 2011, Suda et al. 2007a), where a high speed and large volume tail vein injection of pDNA causes the liver to more than double in size (Suda et al. 2007b). Delivery of a large volume of liquid into the confined tissue space of the liver results in hydrostatic pressures that create temporary pores in the plasma membranes of cells, thus increasing permeability and heightening transfection. In animal models this facilitates gene expression in large numbers of cells in the liver and detectable levels of gene expression in other internal organs. This technique has also been transferred to animal skin, where rapid intradermal injection of a large volume (up to 100µl) of pDNA solution into a mouse foot pad facilitates significant gene expression (González-González et al. 2010). González-González et al. suggest that the presence of nucleic acid in the epidermis is not enough to facilitate keratinocyte uptake and propose that the hydrodynamic pressure created by intradermal injection of a relatively large volume of liquid into the confined space of the mouse foot pad causes transient membrane disruption of skin cells and facilitates cell uptake and subsequent plasmid expression. To date, hydrodynamic delivery has not been successfully used to facilitate transfection in human skin.

In human skin conventional intradermal injection of a pDNA solution also results in observable expansion of the skin tissue. However there is limited gene expression and this is restricted to the point of injection and/or the needle track (Figure 8A-C; E-F). The extent and pattern of transfection following conventional injection is therefore not characteristic of a hydrodynamic effect and is more likely caused by direct jet injection at the needle orifice and/or temporary mechanical damage to the cells at the point of needle insertion. The pressure created in the human tissue by conventional intradermal injection of 50 µl of a pDNA solution is therefore not great enough to facilitate extensive transfection in the cellular region of the skin. However delivery of the same injection volume (50 µl) containing the same reporter plasmid (pCMVB) to the same human skin model using the hollow MN device results in a significant enhancement to the level of exogenous gene expression (Figure 3). It is therefore the specific delivery system i.e. the hollow MN that is responsible for the increase in gene expression witnessed in this study.

Real time OCT videos and supportive microscopy images (Figure 7 & Suppl. Figure 1A-C) indicate that hollow MN delivery of the liquid bolus is more superficial than that of conventional intradermal injection. This results in multiple microdisruptions to the skin architecture in the papillary dermis and at the epidermal-dermal junction (Figure 7 and Suppl. Figurures 1A-C). These disruptions were created during the MN injection process (Figure 7B and Suppl. Figure 1B) but remain following completion of bolus delivery (Figure 7C and Suppl. Figure 1C). The disruptions were located at numerous points that are distant from the point of MN injection but within the confines of the bleb. Some of these changes were also co-localised with points of transfection (Figure 7D). These disruptions were not observed upon conventional intradermal injection (Figure 7A and Suppl. Figure 1A). We therefore postulate that, unlike conventional intradermal injections, hollow MNs are able to force large volumes of liquid (relative to the skin volume) at high speed (typically less than few seconds to administer 50 µl) into the papillary dermis, where elastin and collagen fibres are tightly packed and are in direct contact with the viable cells of the epidermal basement membrane. This creates hydrostatic pressures that physically disrupt the local tissue architecture and cause a transient increase in the permeability of cells in this region, which results in notable gene expression located exclusively with the skin 'bleb' (Figure 3 & 4). This proposed mechanism of transfection is akin to the hydrodynamic delivery witnessed in animal models, the main difference being that we have used a hollow MN device to replicate this in human skin tissue and have achieved it using relatively low injection volumes (50 µl).

The pattern of gene expression following hollow MN mediated delivery of pDNA was not uniform. Discrete areas of gene expression were observed, predominantly in the viable epidermis but also in the dermis (Figure 3-5). The disparate pattern of gene expression is unsurprising given the proposed mechanism of transfection, i.e. hydrodynamic delivery is unlikely to create a uniform pattern of disruption in a heterogeneous tissue architecture. Indeed, OCT images and histology (Figure 7) support this, highlighting discrete areas of mechanical tissue disruption in the papillary dermis and epidermis, rather than uniform tissue disruption. Lateral application of a MN device (Mikszta et al. 2002) was used in the human skin model as an alternative method to create extensive superficial physical disruptions in the viable epidermis and papillary dermis (Figure 8 D, G-H). Delivery using this method also produced a random pattern of discrete areas of gene expression proximal to those points of mechanical disruption. Whilst lateral microneedle application is not

clinically useful, as it causes significant mechanical damage to the stratum corneum barrier, it provides an interesting comparator and supports the hypothesis that intradermal delivery of a pDNA solution through the MicronJet system also physically disrupts cell membranes in the papillary dermis and viable epidermis by a hydrodynamic effect, in this case without compromising the protective stratum corneum barrier.

Transfection in the excised human tissue is therefore considerable following pDNA delivery by the MicronJet, but when skin cells are subsequently extracted from the bleb the relative number of transfected cells is low (Figure 5). This is likely a compound factor of the inherent difficulty in transfecting the dominant cell type of the epidermis i.e. primary terminally differentiating cells (Figure 2), restricted access of pDNA to all cells within the three dimensional skin architecture and, potentially, damage to transfected cells during extraction from the tissue. Successful transfection also relies on transient disruption of the plasma membrane, but in a proportion of cells the physical disruption is likely to be permanent and thus cause cell death. Therefore whilst the data provides an insight into the numbers of cells transfected it is likely to be an under-estimate of the transfection efficiency i.e. of the number of cells expressing the exogenous gene as a proportion of those cells that are able to be transfected. Additionally, levels of localised gene expression are significantly greater than that achieved by conventional intradermal injections or other MN delivery technologies and therefore may be suitable where high transfection efficiencies are not required e.g. nucleic acid vaccines or immunotherapy approaches.

The observable enhancement in performance, compared to conventional intradermal injection and solid MNs, encourages development of hollow MN delivery systems for nucleic acid therapies. Whilst electroporation is now being investigated as an adjunct to enhance intracellular delivery of pDNA following the application of solid MNs (Wei et al. 2014), the hydrodynamic mechanism of cellular delivery associated with use of the hollow MN device may provide a simpler alternative. To the best of our knowledge, this is the first time that hydrodynamic gene delivery has been achieved in human skin and therefore further work is now required to optimise delivery and translate these results from reporter genes to therapeutically relevant pDNA. The extent and reproducibility of exogenous gene expression in human skin indicates that the CE marked MicronJet device may therefore provide a valuable platform for nucleic acid vaccination strategies or immunotherapy approaches. It is also important

to note that whilst multiple sub-surface microdisruptions created by the MicronJet in the skin are able to facilitate cellular uptake and subsequent expression of pDNA, there have been thousands of MicronJet injections with no reports of associated infection and therefore the enhancement in transfection is not at the expense of the minimally invasive principles of the MN delivery system.

#### Conclusions

This study exemplifies, for the first time, the use of hollow MNs for efficient vectorless delivery and expression of pDNA in human skin and proposes hydrodynamic delivery as the mechanism for enhanced gene expression, even with a 'standard' injection volume. Hydrodynamic gene delivery has not previously been observed in human skin tissue. The approved MicronJet hollow MN system ensures accurate delivery of a finite dose of exogenous DNA into the skin and provides more efficient and reproducible gene expression compared to solid MN delivery strategies. The delivery method provides potential advantages over other chemical and physical gene delivery methods with respect to cost, simplicity and flexibility of dosing and therefore could be the basis for future nucleic acid based immunotherapies and vaccines.

#### Acknowledgements

This work is part of the EE-ASI European research network (Collaborative Project) supported by the European Commission under the Health Cooperation Work Programme of the 7th Framework Programme (Grant agreement n° 305305).

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Graphical abstract

