Bio-electrosprayed Living Composite Matrix Implanted into Mouse Models

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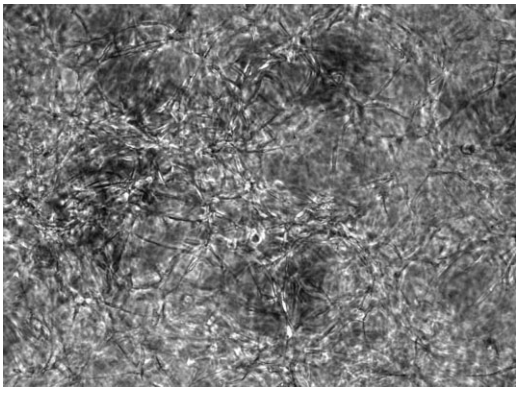
We show that composite de novo structures can be generated using bio-electrosprays. Mouse lung fibroblasts are bio-electrosprayed directly with a biopolymer to form cell-bearing matrices, which are viable even when implanted subcutaneously into murine hosts. Generated cell-bearing matrices are assessed in-vitro and found to undergo all expected cellular behaviour. Subsequent in-vivo studies demonstrate the implanted living matrices integrating as expected with the surrounding microenvironment. The in-vitro and in-vivo studies elucidate and validate the ability for either bio-electrosprays or cell electrospinning to form a desired living architecture for undergoing investigation for repairing, replacing and rejuvenating damaged and/or ageing tissues.



Introduction

Bio-electrosprays,[1–3] invented in 2005, have now conformed to the rigours of scientific method in several respects. In our hands we have addressed the biological and physical features which have seen fundamental questions such as the viability and functionality of the cells assessed from the genomic to the phenotypic level,[4–9] while the physical science elements have seen the fine-tuning of the system for acheiving jet stability and continuity in the number of cells compartmentalised within droplets and residues.[10–13] In parallel to these studies we have also unveiled the ability to directly form cell-laden fibres from which living scaffolds to membranes are directly formed in either a random or pre-organised fibre orientation as a function of depth. This methodology is now refered to as cell electrospinning.[14,15] Additionally, our previous studies have demonstrated the ability to couple these technologies with standard gene therapy protocols for forming cell-bearing therapeutic encapsulations (beads) and scaffolds for controlled and targeted/localised delivery of a wide range of tailor-made therapeutic payloads.[16] These previous studies have also confirmed the ability to directly handle dynamically developing multi-cellular whole organisms.[17,18]These efforts have clearly demonstrated the flexibility of both bio-electrosprays and cell electrospinning for a range of applications spanning tissue engineering and regenerative biology and medicine with advanced cell and molecular-based therapeutics. In the present study we demonstrate the ability to directly form active three-dimensional cell-bearing matrices by combining living cells with a biopolymer using either bio-electrosprays or cell electrospinning (data not shown), which can subsequently

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be implanted into a living host. These first examples demonstrate the promise these biotechniques have for forming a viable composite architecture for repairing, replacing or rejuvenating damaged tissues and, one day, entire organs.

Materials and Methods

Bio-electrospraying

The bio-electrospray set-up employed in these studies has previously been described by us elsewhere.[1,11] Briefly, we explored two bio-electrospray needle systems which varied in configuration, namely either the single or the coaxial system. When the single needle system was utilized, the biopolymer and the cell suspension were mixed and subsequently jetted. In the coaxial configuration, the cell suspension flowed through the inner needle, while the outer needle accommodated the flow of the biopolymer; this system is most useful when forming cell-bearing fibres/scaffolds where the majority of the cells are placed in the central core of the forming fibre.

Cells and Cell Suspension Preparation

The cells used in these studies were primary mouse lung fibroblasts (MLFs), derived from wild-type C57BL/6J mice, and were a kind gift from Professor Shaun Coughlin (University of California, San Francisco, CA). MLFs were maintained in DMEM supplemented with glutamine (4×10^{-3} M), penicillin/streptomycin (100 U $\cdot$ mL^{-1}U), and 10% (v/v) FBS (all from PAA Laboratories, Yeovil, UK), in a humidified atmosphere at 37 °C/10% CO_2. Prior to use, MLFs were removed from the tissue culture flask by standard trypsinization, and subjected to one of three procedures: a) culture controls (CC); cells which have undergone all the cell culture protocols only, b) needle controls (NC); cells that have been subjected to cell culture protocols and made to flow through either needle configuration and then collected (for assessing any needle-based shearing etc) and c) those bio-electrosprayed (BES)/

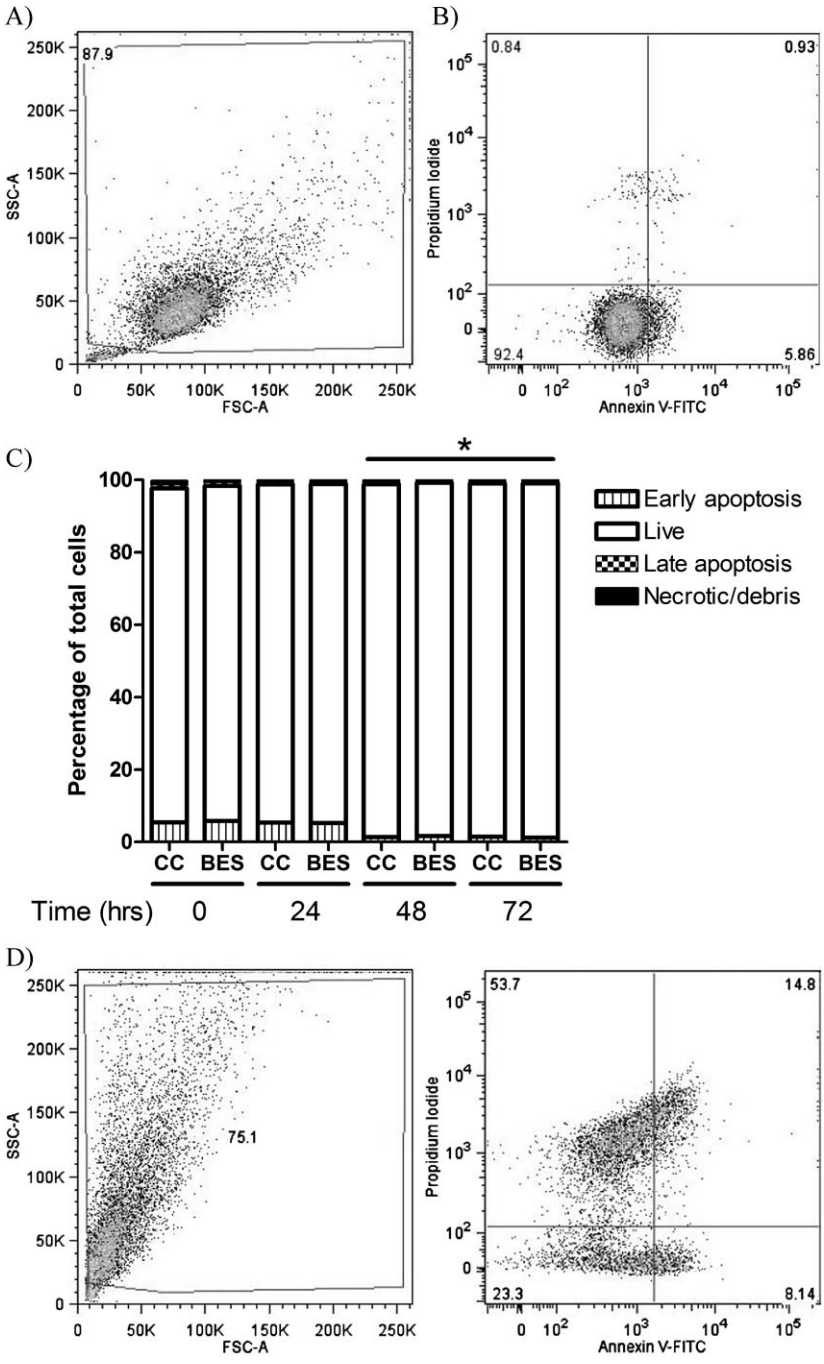

Figure 1. Flow cytometry analysis of cell viability post-bio-electrospraying, using Annexin V binding and propidium iodide staining. A) Representative dot plot for cellular morphology (Forward Scatter and Side Scatter), showing the region selected for assessment of apoptosis. B) Characteristic dot plot for Annexin V binding (x-axis) against PI staining (y-axis); viable cells are in the lower left quadrant, early apoptotic cells in the lower right, late apoptotic in the upper right and dead cells in the upper left quadrant. C) Graphical representation of the mean proportions of live/early apoptotic/late apoptotic/dead cells at each time point for three independent experiments. There was no difference in viability between CC and BES at a given time point. D) Typical dot plot for the positive control of apoptosis, induced by culturing MLF with staurosporine for 24 h. Data analysed by two-way ANOVA with Tukey post-hoc testing; * $p < 0.01$.

cell electrospun (CE). Samples of each CC, NC and BES/CE were assessed for cell viability over a 72 h time frame using flow cytometry (Becton Dickinson LSR II; BD Biosciences, Oxford, United Kingdom). Cells were collected at 0, 24, 48 and 72 h time points and incubated at room temperature for ~15 min in Annexin V buffer containing 1.8×10^{-3} M calcium, 4 μL · mL^{-1} FITC-labelled Annexin V (Pharmingen, UK) and 5 μg · mL^{-1} propidium iodide (PI; Sigma, UK). In parallel, unlabelled cells at these time points were morphologically examined by standard phase contrast light microscopy (Leica MZ10). Labelled samples were immediately analysed using flow cytometry to collect data for >20 000 events for each sample. As a positive control for apoptosis, a separate sample of cells was incubated with staurosporine for 24 h.[19,20]

Preparation of Living Cell-Bearing Matrices and in vivo Implantation

The biopolymer, Matrigel™ matrix,[21,22] derived from the mouse Engelbreth-Holm-Swarm sarcoma (which secretes large quantities of basement membrane components) was purchased from BD Biosciences (Oxford, United Kingdom). Before coming into contact with the Matrigel, all cell culture plasticware was kept at 2 °C for 48 h prior to handling of the biopolymer. Similarly, the bio-electrospray needles were maintained at 2 °C for over 48 h and flushed with fresh medium prior to jetting the composite Matrigel-based cell suspensions. The Matrigel-based cell suspension was prepared by combining ≈1 mL of resuspended MLFs in DMEM at 2 °C with ≈1.5 mL of matrigel. The composite solutions were mixed thoroughly and jetted directly into a chambered 8-well coverslip, with each well having a diameter of 6 mm and a depth of 1 mm (Sigma Aldrich, Dorset, United Kingdom) on sterile glass microslides. The jetted composite cell suspensions in chambered coverslips were incubated at 37 °C/10% CO_2 for ~30 min and later submerged in DMEM/10% FBS for ongoing culture. This protocol was optimised by us in order to generate semi-rigid

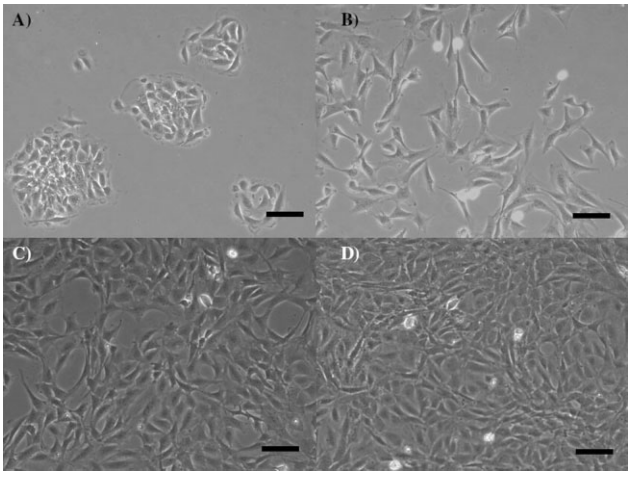

Figure 2. Representative phenotypic studies on post-bio-electrosprayed cells at various time points after jetting; no differences were observed in comparison with controls. Representative images are shown for BES cells after 3 h (A), 24 h (B), 48 h (C) and 72 h (D). The scale bar in all panels represents 100 μm.

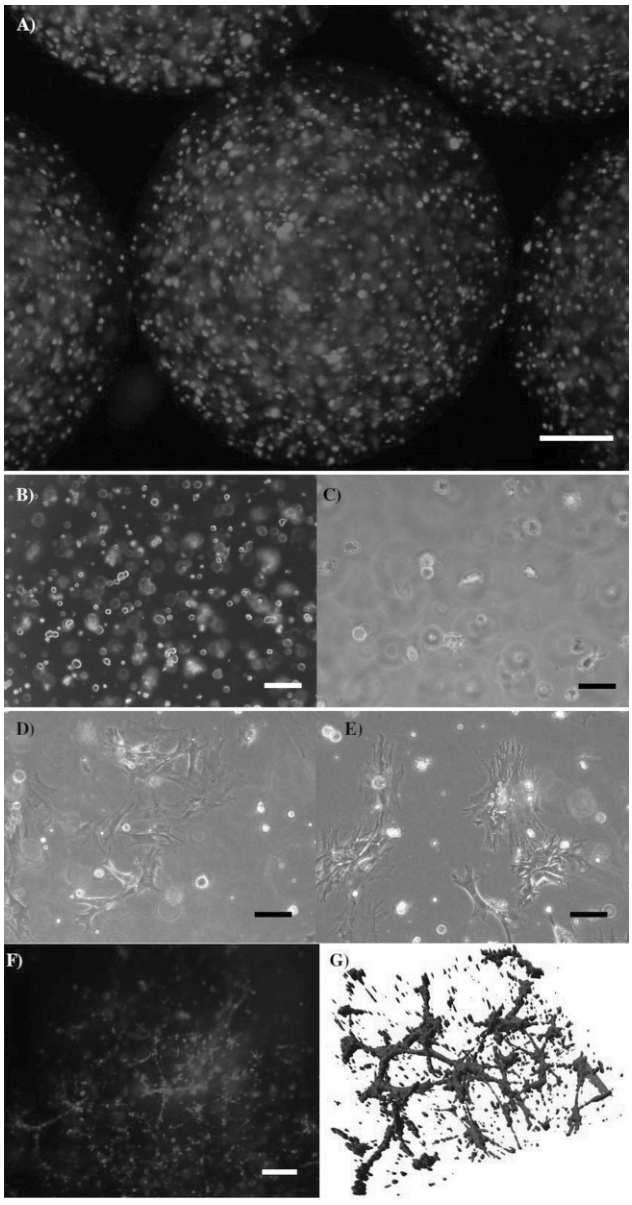

Figure 3. Imaging of fluorescent DiI-labelled MLFs within cell-bearing matrices. Representative fluorescence micrograph depicting homogeneously distributed cells within a CBM immediately after jetting (A). Higher magnification of the same cells under phase contrast (B), and after 24 h (C), 48 h (D) and 72 h (E) of culture. After 72 h, the MLFs reorientate into a complex cellular network which can be easily appreciated by fluorescence microscopy and a three-dimensional reconstruction using confocal microscopy (F and G). The scale bars in panels A) ~1 mm, B) 100 μm, C) 50 μm, D and E) 20 μm and F) 500 μm respectively.

disk-like cell-bearing matrices (CBM) for subsequent implantation.

Male C57BL/6J mice (Harlan, UK) were housed in a specific pathogen–free facility. All procedures were performed on mice between 10 and 12 weeks of age (with 3 mice per experimental group). All animal studies were approved by the University College London Biological Services Ethical Review Committee and licensed under the Animals (Scientific Procedures) Act 1986 (Home Office, London, United Kingdom). Under halothane-induced anaesthesia, the dorsal flank of male C57BL/6J mice was shaved and sterilized at the incision site. An incision (≈5 mm) was made in the skin, and then a small subcutaneous pocket was created by inserting a hemostat into the incision and opening and closing the jaws of the haemostat to spread apart the subcutaneous tissue. CC or BES/CE CBMs created 24 h earlier were then inserted directly into the pocket, and the wound was closed with one or two sutures using 3-O vicryl. Mice were allowed to recover and closely monitored for signs of distress; animals were subsequently sacrificed two days following implantation, and skin encompassing the implantation site was removed, fixed in 4% paraformaldehyde for 24 h, then dehydrated through ethanol prior to embedding in a transverse orientation in paraffin wax. 2 μm sections were then cut for histological analysis using standard Haematoxylin & Eosin (H&E) staining. For control samples, the uninvolved skin from the contralateral flank of a mouse implanted with a CBM was also collected.

In parallel at various time points after formation and subsequent culture, CBMs were removed from the medium, fixed in 70% ethanol for 20 min and then embedded in paraffin wax. For subsequent microscopy, 2 μm sections were then cut and stained with standard H&E. Sections were then visualized by microscopy (DM5000B microscope; Leica Microsystems), and images were captured using a Qicam 12-bit colour fast camera using Q capture software, version 2.81 (both from QImaging Corp.). In parallel experiments, MLFs were preloaded with a cell permeable fluorescent marker (DiI; Invitrogen, UK), and cells within CBMs were observed directly using fluorescent or confocal microscopy.

Bio-electrospray Optimization

Optimisation studies allowed us to fully assess the bio-electrospray characteristics of these cell suspensions in both media and FBS and/or with the Matrigel matrix, with variable cell density and Matrigel matrix concentrations, in order to establish operational parameters permitting the collection of a majority of the cells, jetted directly into the chambered coverslip, whilst minimising cell loss. It was noted that if an applied voltage to flow rate combination of ∼10 kV and a $\approx 10^{-9}$ $m^3 \cdot s^{-1}$ was maintained, jetting would take place in the unstable cone-jetting mode which was shown to directly jet a large majority of cell-bearing droplets into the chambered coverslip with the aid of a combined ground electrode system. Moreover, we demonstrated that increasing the cell density to $\approx 10^7$ cells/mL for a Matrigel:cell suspension ratio of ∼1.5:1 would give better jetting conditions (including trajectory) while minimising cell loss during collection.

Results and Discussion

Bio-electrosprayed CBMs were subsequently split into two groups for: 1) optical/fluorescent microscopy analysis and 2) subcutaneous implantation into mice. Flow cytometry analysis of the CC, NC and BES established that bio-electrospraying under these operational conditions was inert in terms of cell viability in comparison with controls. Data for the direct comparison of CC and BES are shown in Figure 1; no significant differences were observed in terms of cellular morphology as assessed by forward and side scatter (Figure 1A), nor in the proportion of live

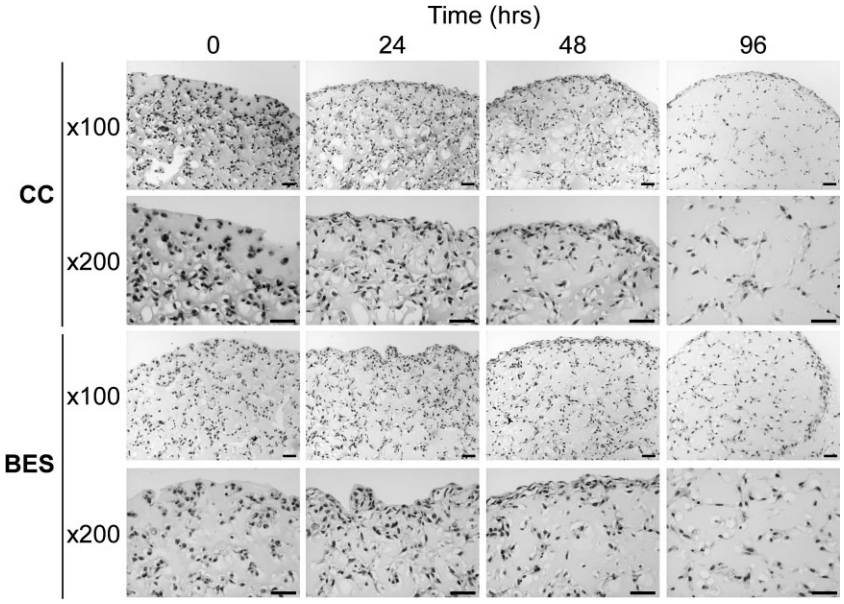

Figure 4. Histological analysis of paraffin wax-embedded cell-bearing sections having a thickness of 2 μm. CBMs equivalent to those described in Figure 3 were fixed and embedded for sectioning and standard histological analysis using H&E staining. There were no observable differences between CC and BES cells at any time point studied, and the formation of cell networks beyond 72 h of culture can be appreciated. Images were taken at an original magnification of x100 and x200. Scale bar: 50 μm.

cells when comparing CC and BES at a given timepoint (two-way ANOVA with Tukey post-hoc testing; Figure 1B, C). However, both CC and BES had a significant increase in the proportion of live cells at 48 and 72 h post-treatment, compared with the earlier timepoints, which likely reflects the proliferation of viable cells and hence the diminution in proportion of detectable dead/dying cells (Figure 1C). In contrast MLFs treated with staurosporine for 24 h, as a positive control for apoptosis, showed a clear and dramatic increase in the proportion of dying/dead cells (Figure 1D) as expected.

In conjunction with flow cytometric analysis, phenotypic studies of the cells were carried out optically over a period of 72 h and again, no significant differences were observed between cells bio-electosprayed versus controls (Figure 2). A similar phenotypic assessment was carried out histologically on samples which were generated by jetting cell suspensions containing matrigel. At early time points after formation, both CC and BES cells were homogeneously distributed throughout the matrigel scaffold (Figure 3 and 4). However with increasing time in culture (particularly evident beyond 72 h), both CC and BES CBMs were seen to initiate interconnecting cellular networks rather than a random scatter. This is also clearly appreciated in fluorescence micrographs of entire CBMs captured immediately after jetting (Figure 3A), and by confocal imaging of the cellular networks 72 h after culture (Figures 3F & G). Thus, bio-electrosprayed cells demonstrated no unexpected cellular behaviour whilst being encapsulated in three-dimensions, and indeed retained the capacity to migrate and remodel within their local environment (Figures 3 and 4). The reader should note that in all our experiments the needle controls were indistinguishable with those culture controls.

Having established that CC and BES-generated cell-bearing matrices were entirely equivalent in terms of cell viability and functionality *in vitro*, the potential for *in vivo* implantation was then investigated. None of the implanted mice suffered any obvious ill effects (no piloerection, decrease in mobility or abnormal posture) during the period of implantation; weight loss was minor and consistent with that normally seen post-surgery. There was no significant difference in percentage weight loss between the mice given CC or BES CBMs at two days post implantation (1.7% $\pm$1.6% in the CC group vs 3.6% $\pm$0.4% in the BES group; n = 3 per group).

In contrast to control skin (Figure 5A), after two days CBMs were still clearly evident within the subcutis layer of the skin (Figures 5B-E). MLFs were observable within the Matrigel scaffold; in addition, a very mild inflammatory cell infiltrate was present surrounding the CBM, consistent with the effects of surgical implantation. As with our *in vitro* studies, there was no observable phenotypic difference between CC and BES-treated CBMs, demonstrating that bio-electrospraying does not significantly impair the potential for in vivo engraftment of cell-bearing matrices.

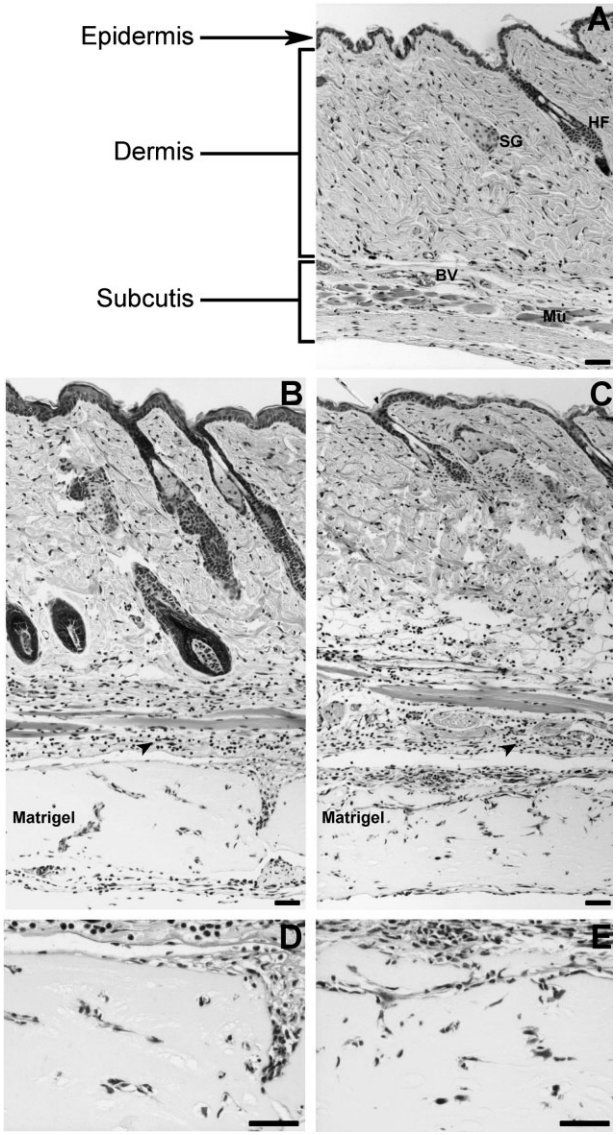

Figure 5. Subcutaneous implantation of cell-bearing matrices into mice. CC or BES-treated CBMs were inserted subcutaneously into the dorsal flank of C57Bl/6J mice. The skin was harvested and sectioned for histological analysis after 2 d. H&E staining of control mouse skin is shown in (A), demonstrating the normal architecture of the epidermis, dermis and subcutis layers (original magnification x100). Following implantation, CBMs (labelled 'Matrigel') were observable in the subcutis layer of mice receiving CC and BES-treated CBMs (panels B and C respectively; original magnification x100), with occasional inflammatory cells in the surrounding tissue (arrowheads). Higher magnification micrographs of MLFs within the Matrigel scaffold are shown in D and E (original magnification x200). HF, hair follicle: SG, sebaceous gland; BV. blood vessel; Mu, muscle. Scale bar: 50 μm.

Conclusion

These studies demonstrate the ability for bio-electrosprays and cell electrospinning to directly handle and form composite fully functional three-dimensional cell-bearing matrices, which can subsequently be implanted into mouse models. Having established that these technologies could be further explored for enabling repair of the largest organ, namely skin, the authors intend to take advantage of these findings for targeting other intricate organs such as lungs, heart, kidneys etc. In a parallel investigation but where aerodynamically assisted bio-jetting (AABJ) was explored for forming CBM's, similar results were established. With future refinements, we can envisage such active matrices being pursued for not only repairing, replacing and rejuvenating damaged or aged tissue in-situ, but also for the delivery of a therapeutic payload using a cell-based gene therapy approach.[23]

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