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Ex Vivo Transduction of Human Dermal Tissue Structures for Autologous Implantation Production and Delivery of Therapeutic Proteins

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Systemic delivery of therapeutic proteins through gene transfer approaches has been carried out mostly by *ex vivo* transduction of single cells or by direct *in vivo* injection of an expression vector. In this work an intact miniature biopsy of human dermis (microdermis) is harvested and transduced *ex vivo* by a viral vector encoding a gene for the therapeutic protein. The microdermis preserves its three-dimensional structure and viability during the *ex vivo* manipulations. Furthermore, upon transduction with adenoviral and adeno-associated viral vectors the microdermis secretes recombinant human erythropoietin (hEPO). Biochemical analysis of the secreted hEPO showed similarity to the clinically approved recombinant hEPO. Subcutaneous implantation of microdermal hEPO into SCID mice exhibited hEPO secretion in the blood circulation and preserved elevated hematocrit for several months, demonstrating the technology's potential for sustained delivery of protein therapeutics.

INTRODUCTION

Recombinant therapeutic proteins have been used clinically for more than 2 decades. In general, these protein drugs are manufactured in cultures of nonhuman cells and then purified, formulated, and administered mainly by intravenous or subcutaneous bolus injections. Due to their short half-life in the circulation, treatment duration is achieved by administering large bolus doses, which results in exceeding optimum therapeutic serum levels and causing possible adverse clinical outcomes [1]. These serum concentrations rapidly decline to below therapeutically effective levels within hours to days, requiring repeated injections to maintain the therapeutic effect [2]. Thus, industrial production and bolus administration of therapeutic proteins is both inefficient and clinically undesirable. One such protein therapeutic is human erythropoietin (hEPO), a 165-amino-acid polypeptide containing two disulfide bridges and four glycosylation sites [2], which is produced mainly by the kidney. The major biological function of EPO is to regulate the number of committed erythroid precursors by rescue of those precursors that would otherwise undergo apoptosis and drive their maturation toward mature erythrocytes. Thus, it is EPO's constant presence above a specific threshold, and not high serum levels of EPO, that is critical for treatment efficacy [2].

Gene or cell therapy provides the potential for natural, continuous production and delivery of therapeutic proteins using human cells. However, systemic or local administration of some viral vectors, and in particular adeno vectors of the first generation, was found to elicit immune response. Furthermore, it has been difficult to dose or control drug levels *in vivo* [3]. Genetically modified cell therapy approaches are limited by shortcomings in cellular encapsulation technologies and by complicated *in vitro* cultivation of primary cultures [4]. An alternative approach to the use of single cells is the application of three-dimensional micro-organs of solid tissues as was shown recently by Grad-Itach *et al.* [5]. Micro-organ culture of rat liver maintained normal functions *ex vivo* and when placed in an extracorporeal device was able to increase survival of hepatectomized rats. Based on this concept, we have developed an intact microdermal tissue structure that is genetically manipulated *ex vivo* to promote, upon reimplantation, continuous production and delivery of therapeutic recombinant proteins.

The microdermis technology is based on a defined harvesting and *in vitro* maintenance procedure that

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enables long-term *in vitro* preservation of the dermal structures. Fig. 1 illustrates a schematic representation of the microdermis system: (a) Following harvesting of the microdermis the three-dimensional tissue is placed in culture. (b) Viral vector is applied for cellular transduction and remaining viral particles are removed by extensive washing. (c) An ELISA is used to quantify secretion of the recombinant protein, providing a tool for establishing the optimal number of microdermis units required to achieve a therapeutic dose, and the microdermis is then implanted subcutaneously in the patient's skin. There, it integrates and functions, to deliver the therapeutic protein to the patient's circulation. The microdermal protein production is reversible since the implanted microdermis remains localized and accessible and can be ablated, if needed.

RESULTS

Viability of Microdermis Tissue Maintained *ex Vivo*

We harvested microdermis units from abdominal human skin samples via a rotating coring needle and genetically manipulated and maintained them *ex vivo.* The harvesting and maintenance protocols have been optimized to preserve the dermal tissue's natural structure and viability while producing sufficient quantities of hEPO upon transduction. Infection with Ad5-hEPO or AAV1/2-hEPO viral vectors results in different secretion levels and kinetics (Fig. 2a). While the adeno vector gave high EPO expression early on that declined rapidly within several weeks, expression following transduction by the AAV vector steadily increased even after 120 days in culture. This demonstrates that transduced microdermises are viable and continuously secrete hEPO for extended periods of time while maintained *ex vivo.* However, in practice, microdermises are produced and implanted within 9 days postharvest. Therefore, we determined tissue viability by assaying the culture medium for metabolic consumption of glucose, as well as by histological analysis for signs of tissue decay during the *ex vivo* culture period. Multiple microdermises produced from different donor skin samples remain viable during the *ex vivo* maintenance period, as demonstrated by similar glucose consumption after 5 and 8 days in culture (Fig. 2b). In contrast, microdermal cultures that underwent two freeze/thaw cycles on day 5 after harvesting showed little or no glucose consumption, indicating cell death (Fig. 2b, sample K*). Furthermore, histology of the microdermis during the *ex vivo* culturing on days 1 and 9 after harvest (Fig. 2c) indicates no major changes in dermal structures, such as collagen fibers. In addition, both blood vessels and sweat glands are visible during the *ex vivo* manipulation (Fig. 2c). Analysis for cell apoptosis during the *ex vivo* culturing of the microdermis, using the *in situ* TUNEL histology assay, also did not reveal cell death (data not shown). Taken together, these data provide evidence that the microdermis remains viable in culture and consequently is able to secrete hEPO continuously for long periods of time.

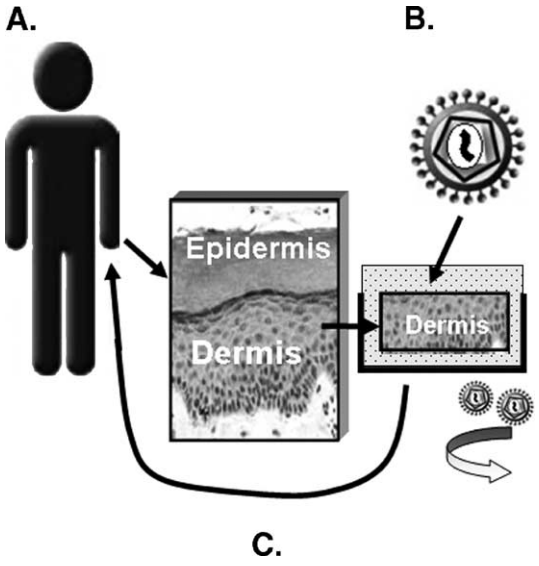

FIG. 1. Scheme of the microdermis technology. (A) A miniature dermal structure (approximately 30 × 2 mm) is removed from the patient's skin using a specialized device. (B) Viral vectors transfer the gene into dermal cells and remaining viral vectors are removed by extensive washing. Following the transduction procedure the desired protein is secreted into the culture medium and quantified by ELISA. (C) The measured protein secretion rate is used to estimate the number of autologous microdermises to be implanted to provide the desired therapeutic protein level in the patient. Following sterility and viability tests the microdermis is reimplanted subcutaneously using an injection-like procedure. There, it integrates as normal tissue, delivering the therapeutic protein to the patient.

Recombinant Protein-Producing Cells and Microdermal Secretion Levels

The dermis is a fibrous matrix containing different structures and several types of cells [6]. In contrast to other more compact tissue structures, the dermal fibrous organization allows for direct viral vector penetration that is independent of vascular perfusion and, thus, for high cellular transduction efficiency *ex vivo.* Histological analysis of microdermis transduced by Ad5-hEPO, using anti-hEPO immunohistochemistry, demonstrated cellular transduction (brown stained cells) throughout the tissue (Fig. 3a, top). Fibroblast-specific immunostaining of consecutive histology sections from the same specimen, using vimentin protein as a marker, showed that many of the hEPO-producing cells are fibroblasts (Fig. 3a, middle). Interestingly, we find that most of the transduced cells are dispersed along either dermal fibers or surrounding structures like hair follicles. Almost no transduction is seen within

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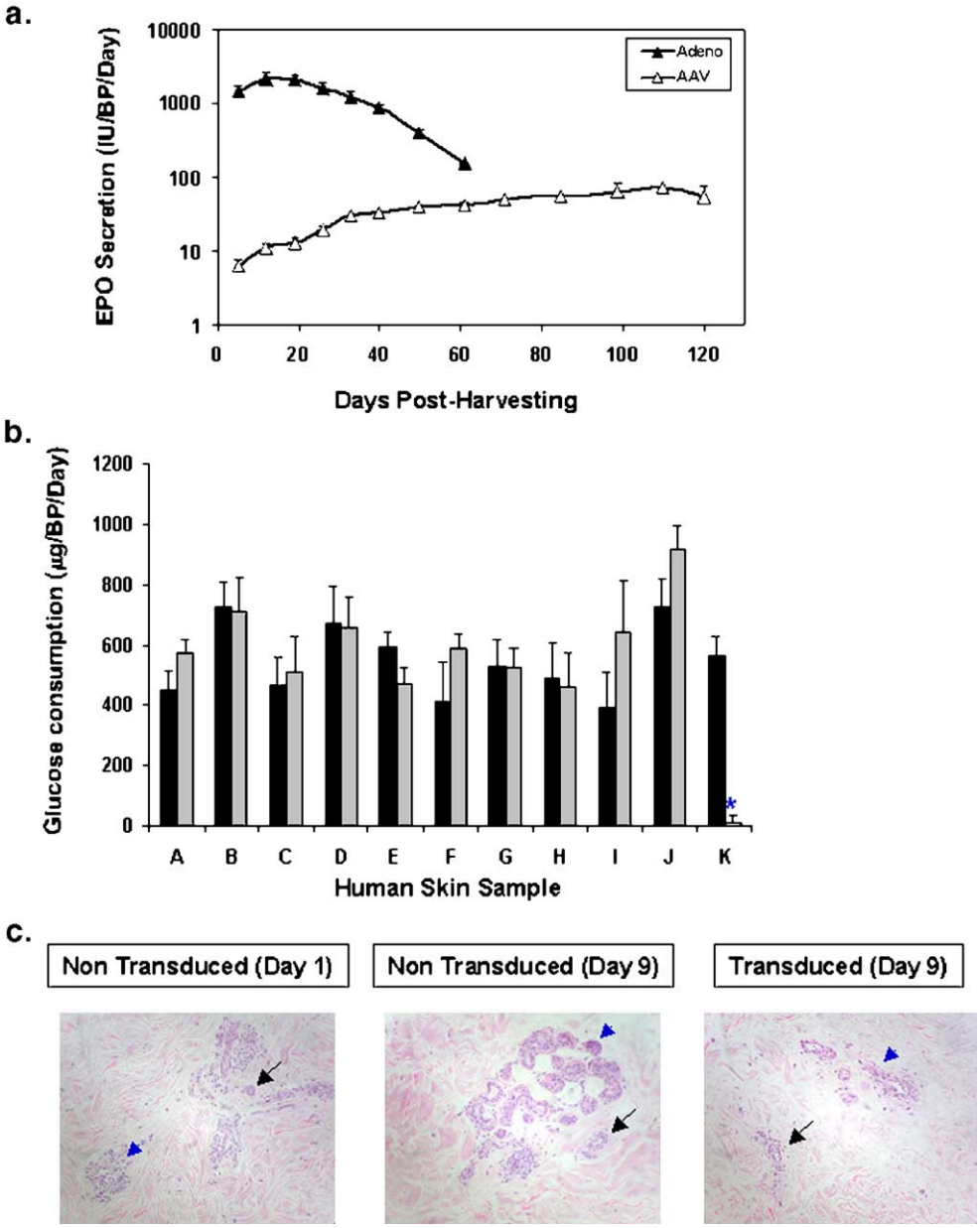

FIG. 2. The microdermis remains viable during *ex vivo* maintenance and secretes hEPO. (a) The levels and duration of hEPO secretion were compared between microdermises transduced with Ad5-hEPO (black triangles) or AAV1/2-hEPO (white triangles). (b) The microdermis metabolic consumption of culture medium glucose was measured on days 5 (black bars) and 8 (gray bars) postharvest. The glucose consumption of microdermis harvested from multiple human skin samples remains stable over time, indicating that the dermal cells remain metabolically active *ex vivo.* As a control (sample K), microdermis that underwent freeze/thaw on day 5 (*) showed little or no glucose consumption by day 9 (data presented as average (SD) of five to seven microdermises). (c) H&E staining of 4-μm sections from different nontransduced microdermises on day 1 and day 9 postharvest, in comparison to an Ad5-hEPO-transduced microdermis on day 9 postharvest. The histological analysis shows that the dermal tissue retains its structures during *ex vivo* maintenance and transduction, with no apparent signs of tissue necrosis (blue arrowheads indicate skin glands and black arrows indicate blood vessels).

glands or blood vessels, presumably due to limited viral penetration into these structures. A nontransduced microdermis control that was sectioned and stained for hEPO shows no background staining of dermal cells (Fig. 3a, bottom).

The transduction efficiency of the microdermis determines the production and secretion of hEPO quantities. To assess the daily hEPO secretion capacity, we produced transduced microdermises from multiple human abdominal skin samples and measured the daily hEPO secretion on the day before implantation (day 8 postharvest). As can be seen in Fig. 3b, the daily secretion of EPO from a microdermis is on average above 1000 IU/day, and a skin from one patient gave up to 2000 IU/day. These levels correspond to approximately 8–16 μg of hEPO protein per day (the specific activity of the standard hEPO is 0.124 IU/ng). This level of hEPO secretion corresponds to the therapeutic amounts of hEPO typically injected into patients [2].

Moreover, we find relatively low variability in secretion levels between microdermises produced from the same individual skin sample, while variability among different donor skin samples is more notable (data not shown).

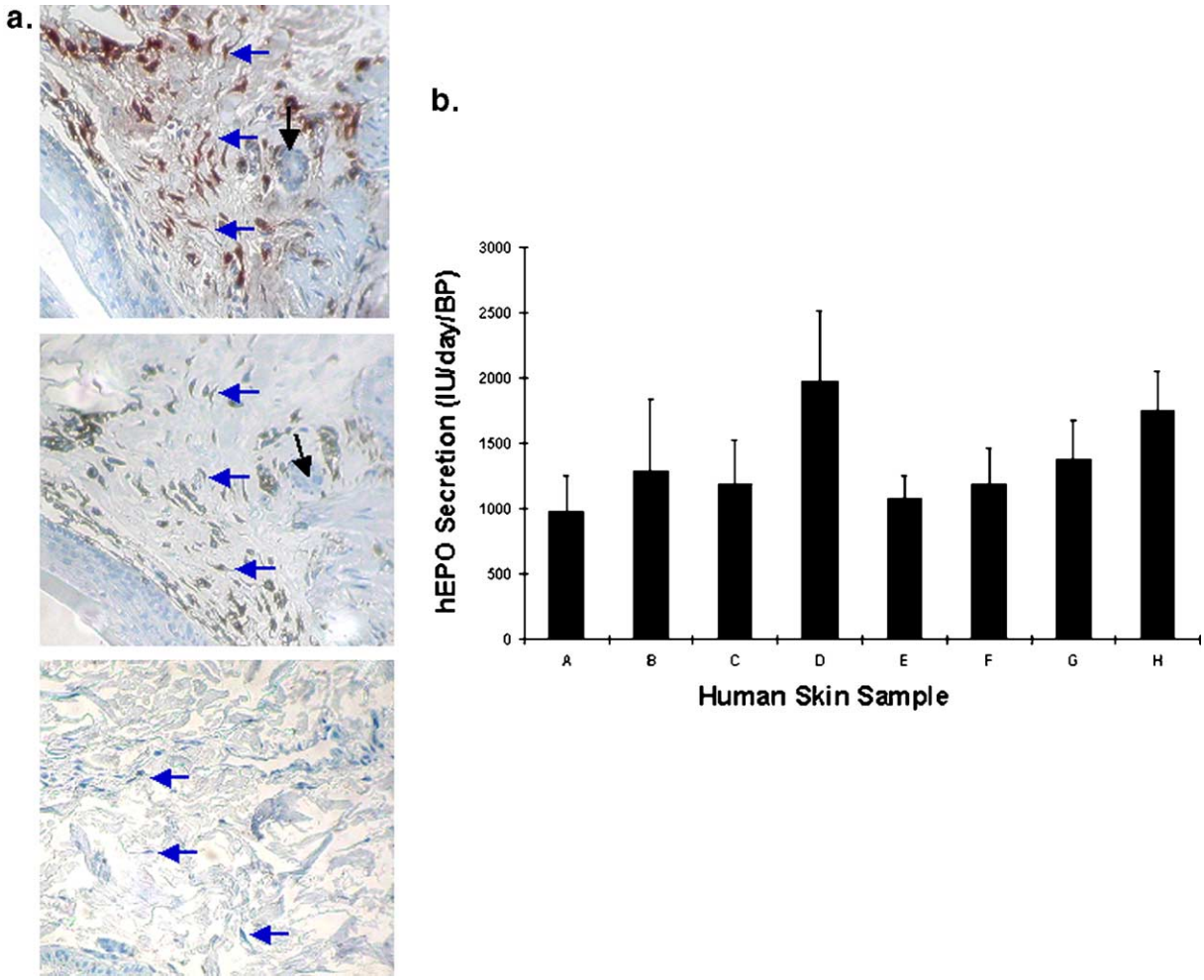

FIG. 3. Therapeutic levels of hEPO are produced by fibroblasts within the dermis. (a) Histology. 4-μm sections of microdermis transduced with Ad5-hEPO were immunostained for hEPO (top) and for vimentin, a fibroblast-specific protein marker (middle), on day 9 postharvest. Many of the hEPO-producing cells are fibroblasts (blue arrows), which are spread throughout the dermis (blood vessels are noted by black arrows). A nontransduced microdermis control section, treated with anti-EPO, shows no background staining (bottom). (b) Microdermises were produced from multiple human abdominal skin samples (A–H) and hEPO secretion levels by individual microdermises were measured by ELISA on the day before implantation (day 8 postharvest). Four microdermises of each patient were tested and the results are given as averages (SD).

Characterization of Microdermal hEPO Recombinant Protein

To verify that the protein secreted by the microdermises is indeed human erythropoietin, we purified it from the conditioned medium and characterized it using multiple biochemical assays. Although microdermal hEPO protein migrated somewhat slower and had less diffused banding, its size (~40 kDa) is similar to that of the standard Biological Reference Preparation (BRP) recombinant hEPO (Fig. 4A). Furthermore, the secreted protein reacts with anti-hEPO monoclonal antibodies in both ELISA and Western blot analyses. The broad protein band represents a variety of glycosylations and is common to hEPO [7]. In fact, similar to the microdermal hEPO the endogenous hEPO, isolated from urine, demonstrates a somewhat higher apparent molecular weight compared with the recombinant hEPO produced in Chinese hamster cells [2]. Column chromatography (HPLC) was performed on microdermal hEPO and on the standard (BRP) rhEPO (Fig. 4B). We analyzed the secreted hEPO fraction within the crude preparation (14.26 min, denoted by arrows) that had a retention time similar to that of the standard BRP rhEPO by N-terminal sequencing and mass spectrometry. N-terminal sequencing confirmed that the first 15 amino acids were identical to the sequence of the endogenous hEPO. In addition, mass spectrometry analysis showed that the peptide amino acid sequences of microdermal hEPO protein correspond to the expected amino acid sequence of hEPO. Taken together, these results indi-

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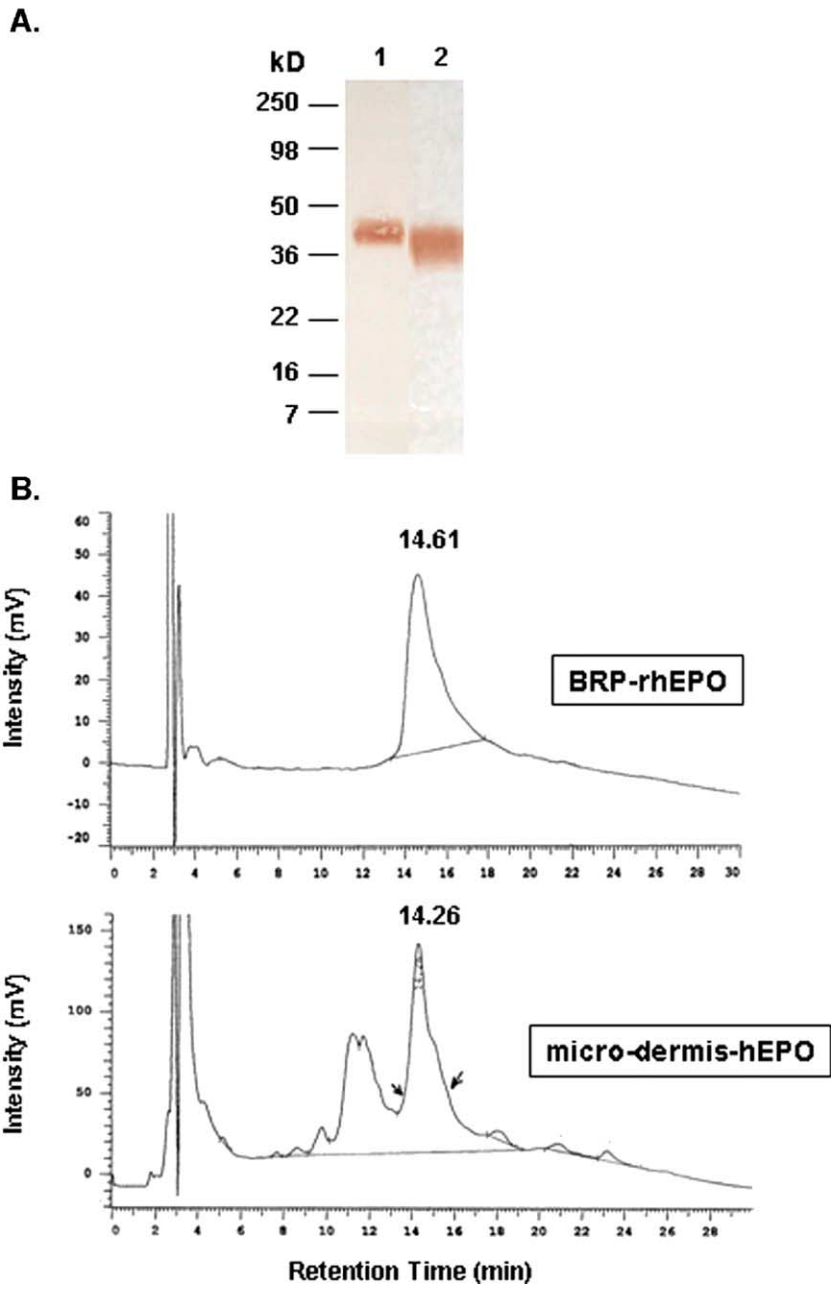

FIG. 4. Biochemical characterization of hEPO protein secreted by microdermises transduced with Ad5-hEPO. (A) Western blot of a microdermis culture medium sample representing 25 ng of hEPO (lane 1) and 25 ng of standard BRP rhEPO (lane 2). (B) Chromatogram comparing the retention time of standard BRP rhEPO (top) to that of microdermis hEPO (bottom) on reverse-phase HPLC. The fractions taken for further analysis are marked by arrows.

cate that the protein secreted by the microdermis is human erythropoietin.

Dose Response and Biological Activity of Microdermal hEPO

The ability to establish quantitatively the recombinant protein secretion capacity per day of the microdermis before implantation is essential for treatment dosing in a clinical scenario. To assess the correlation between preimplantation *in vitro* secretion levels and serum levels following microdermis implantation, we subcutaneously implanted Ad5-hEPO-transduced microdermises secreting different daily amounts of hEPO *ex vivo* into SCID mice and assayed the resulting hEPO serum levels several days postimplantation. As can be seen in Fig. 5A, there is a good correlation (r^2 = 0.6726) between the *in vitro* preimplantation daily secretion levels and the circulating serum levels postimplantation of individual microdermises, indicating that treatment dosing is feasible. In addition, in experiments in which the

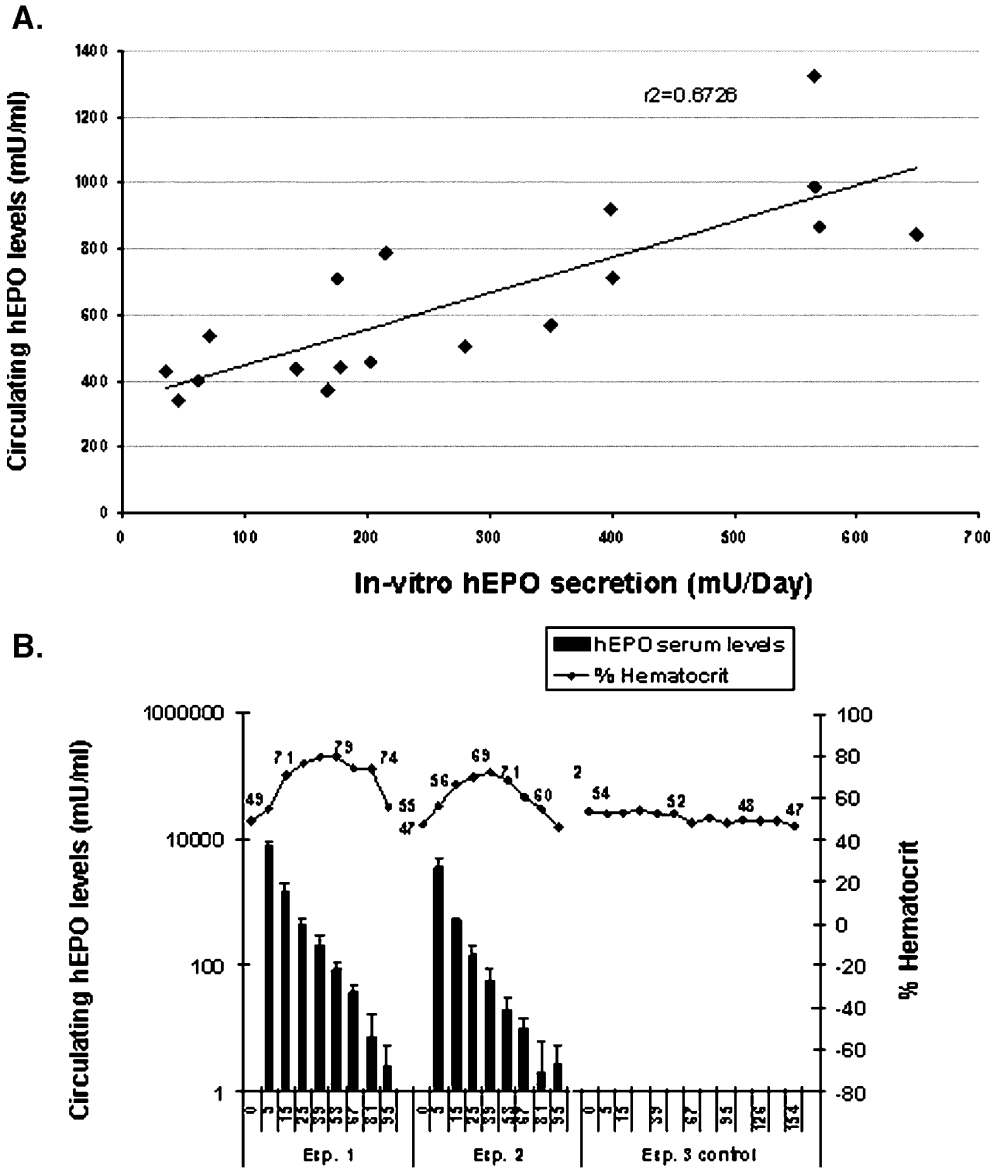

FIG. 5. (A) Correlation between microdermis hEPO secretion levels *ex vivo* and the resulting *in vivo* hEPO levels in the serum after implantation of SCID mice. Microdermises were transduced with Ad5-hEPO and SCID mice were implanted subcutaneously with two hEPO microdermises per mouse. The hEPO secreted by the two hEPO microdermises after 8 days in culture was compared to the *in vivo* hEPO serum levels, assayed on days 15–17 following implantation. Data were collected from four experiments (total of 18 implanted mice). (B) Implantation of hEPO microdermis in SCID mice demonstrates hematopoietic activity. Microdermises were transduced with either Ad5-hEPO or Ad5-LacZ (a negative control) and after 9 days two microdermises were implanted per SCID mouse in two independent experiments (5 mice in each experiment). The serum hEPO levels in the mice at different time points (bars) and the corresponding hematocrit values (lines) were monitored. No hEPO levels could be detected in the serum of an animal implanted with Ad5-LacZ.

microdermal hEPO was either ablated with a radiofrequency needle or surgically removed from SCID mice, we have shown that the treatment is reversible (data not shown).

Implantation of human microdermis secreting hEPO into SCID mice results in a rapid elevated serum hEPO, erythropoietic activity, and a consequent increase in the mouse hematocrit, which can be maintained for up to 2 months (Fig. 5B). We implanted the control group with microdermises that were transduced with Ad5-LacZ. These mice had no hEPO in the circulation and maintained a normal hematocrit of approximately 50%. GLP toxicological analysis of SCID mice 1 month following microdermal-hEPO implantation, which delivered doses that are 500–700 times higher than the mean human dose, showed no significant adverse events (data not shown). As seen in Fig. 5B, we observed a significant decline in hEPO secretion levels after several weeks when using the Ad5-hEPO vector. We therefore prepared microdermises transduced with AAV1/2-hEPO that we subsequently implanted in SCID mice. The results shown in Fig. 6 indicate long-term secretion of hEPO following implantation of microdermis transduced by AAV1/2-hEPO. Implantation with nontransduced control microdermal tissue gave no elevation of hematocrit levels. Interestingly, relatively low levels of steady-state hEPO secretion were required to induce

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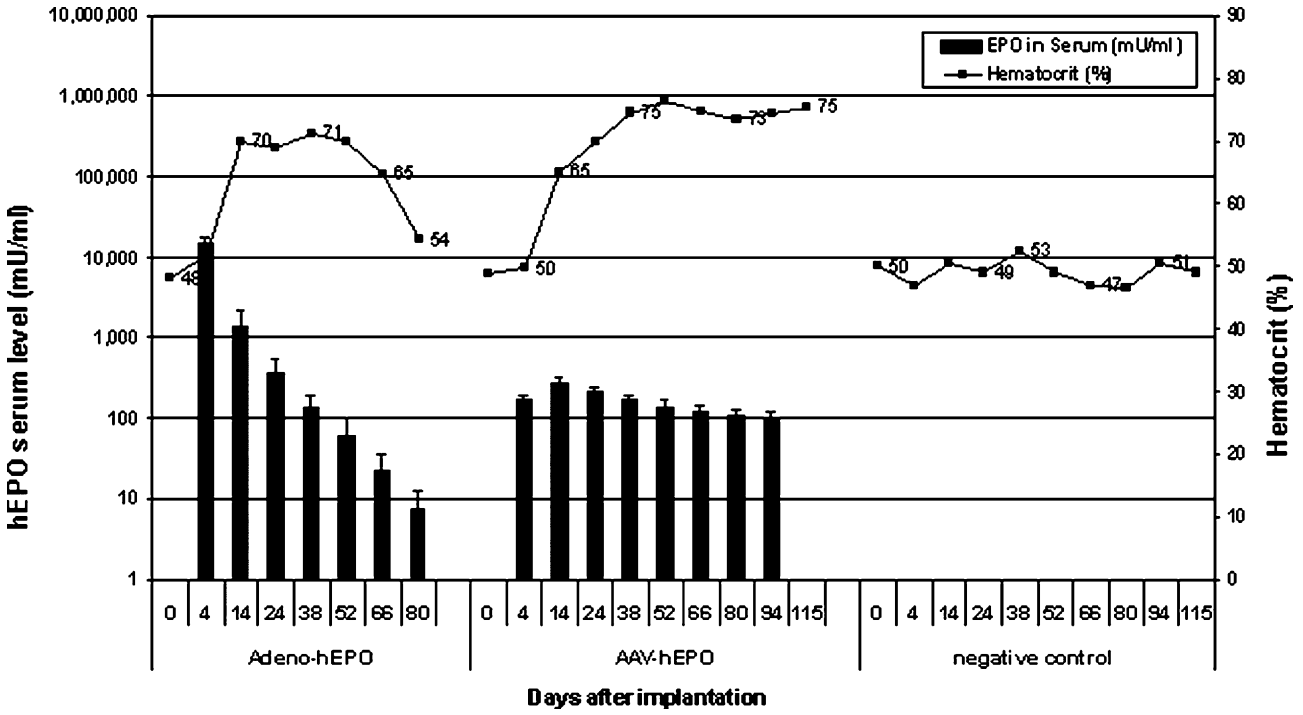

FIG. 6. Secretion of hEPO and hematocrit values in SCID mice implanted with microdermis transduced by AAV-hEPO. Microdermises were transduced with either AAV1/2-hEPO or Ad5-hEPO, as described under Materials and Methods and in the legend to Fig. 2. After 9 days in culture two microdermises were implanted per SCID mouse (5 mice in each group). The hEPO serum levels in the mice at different time points (bars) and the corresponding hematocrit values (lines) were monitored. Control mice (n = 5) were implanted with nontransduced dermal tissue.

continuous elevation of the hematocrit in the SCID mice for at least 115 days.

DISCUSSION

Here we present the microdermis system as a novel and practical approach to therapeutic protein production and delivery using the patient's own skin. We show that miniature human dermal biopsies, of approximately 30 mm in length and 2 mm in width, remain viable during *ex vivo* maintenance for extended periods of time, allowing for tissue genetic manipulation and protein drug quantification before autologous reimplantation. We find that such miniature dermal structures have the capacity to produce therapeutic quantities of hEPO that is similar to the injected standard recombinant protein and active in animal models.

The skin has been previously investigated as a potential production and delivery site of therapeutic proteins through different gene and cell therapy approaches, such as direct injection of viral vectors to the skin [8,9] or subcutaneous implantation of encapsulated producer cells [10–13]. The microdermis concept offers several advantages over conventional gene and cell therapy: (a) The dermal layer of the skin is accessible for relatively simple subcutaneous tissue harvesting and implantation; (b) the fibrous structure of the dermis facilitates excellent vector tissue penetration, leading to high transduction efficiency; (c) *ex vivo* transduction and the extended maintenance period allow for removal of excess viral vectors and intracellular degradation of viral capsid proteins before implantation; (d) preimplantation measurement of the microdermis recombinant protein secretion capacity allows for pretreatment dosing; (e) the recombinant protein-expressing cells are already situated within their natural dermal matrix throughout the preparation period, allowing for the development of fully mechanized manufacturing and avoiding the need for cell purification, expansion, or encapsulation; and (f) the implanted microdermis is localized and accessible for subcutaneous ablation, if there is a need of treatment reduction or discontinuation. These practical advantages make the microdermis concept attractive and, in principle, present a platform technology that holds promise for sustained production and delivery of hEPO, as well as other protein therapeutics.

Since quiescent fibroblasts are known to exist for many months in their natural dermal setting [6], they hold the capacity to support microdermis therapeutic protein delivery for extended periods of time. Nevertheless, in this study we observed limited duration of high-level secretion kinetics of hEPO in SCID mice

following implantation of Ad5-hEPO-transduced microdermis (Fig. 5) and similar kinetics in long-term *in vitro* culturing (Fig. 2). Since microdermal tissue viability has been demonstrated both *in vitro* and *in vivo,* the hEPO secretion kinetics observed might be related to the Ad5-CMV-hEPO viral vector. First-generation adenovirus vectors have been shown to maintain long-term secretion *in vivo* following transduction of slow dividing cells [14]; however, the CMV promoter utilized in this study has been shown to be subject to active cellular shutdown in fibroblasts [14,15]. We are currently screening for an alternative promoter and viral vectors that will support long-term protein drug production. In this context, although the levels of secreted hEPO from microdermis transduced with Ad5-CMV-hEPO began to decline within 20 days, transduction with AAV1/2-hEPO resulted in continued secretion of increasing amounts of hEPO for over 120 days *ex vivo* (Fig. 2). This observation further indicated the viability of the engineered dermal tissue over extended culturing *ex vivo*. While microdermis transduced with AAV-hEPO produced relatively low levels of serum hEPO *in vivo,* these amounts appear sufficient to enhance erythropoiesis and subsequently the hematocrit. Thus, it is possible that maintaining a low but steady-state level of hEPO is an important determinant in erythropoiesis.

The daily secretion level of microdermis transduced with Ad5-hEPO should be sufficient for human treatment, since the mean recommended subcutaneous dose of a recombinant hEPO (Epogen) is approximately 50–100 IU/kg three times weekly, which translates to 1500–3000 IU per day for a 70-kg patient [2]. Thus, implantation of one or a few microdermises secreting hEPO should deliver therapeutic hEPO levels in humans.

Materials and Methods

Construction of viral vectors. To construct rAd5-CMV-hEPO (E1/E3 deleted) the human EPO cDNA was inserted into the pAd-lox shuttle vector [15] containing the CMV promoter and SV40 poly(A) site. The LacZ gene was used to construct Ad5-LacZ [15] (the adeno vectors were kindly provided by Dr. Paul Robbins, University of Pittsburgh, PA, USA). Large-scale growth of research grade Ad-CMV-hEPO was performed by amplification on 293 cells and purification by CsCl (QBIOgene, Montreal, QC, Canada). Concentration of the purified virus particles (VP) was quantified by OD and determined to be ~10^{12} VP/ml. Infectious particles (IP) were determined by plaque assay on 293 cells (VP to IP ratio ~5). The AAV1/2-hEPO vector, based on the AAV-2 backbone and the CAG promoter (AAV1/2-SAR-CAG-hEPO-WPRE-BGH-polyA), is a chimera recombinant AAV, containing both the AAV1 and the AAV2 capsid proteins [16]. AAV was purified on a heparin column to a concentration of 1.1×10^{12} VP/ml. The AAV vector was purchased from GeneDetect.com (New Zealand).

Harvesting dermal structures. All procedures involving human skin have been reviewed and approved by the IRB committee of Rambam Hospital, Haifa, Israel. A thin-gauge hypodermic needle was attached to a 1-ml syringe filled with sterile saline. The needle was inserted into the dermis at the harvesting site and advanced the length of the microdermis (30–40 mm) where it exited through the skin surface. This needle was used as a guide for harvesting the dermis. Next, the skin surrounding the guiding needle was pinched with a surgical clamp and a coring needle (1–2 mm in diameter) was placed on the tip of the guiding needle at its exit point. A small amount of sterile saline was injected into the coring needle, which was then rotated by a medical drill (Aesculap Micro Speed, Melsungen, Germany) and advanced manually along the length of the guiding needle, resulting in a 30- to 40-mm dermal core. The dermal core was removed from the guiding needle and placed in a well (24-well plate) containing 1 ml of DMEM production medium, containing 10% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA), high glucose (4.5 g/L), 25 mM Hepes, 1% L-glutamine (Hy-Clone, USP grade), 50 μg/ml gentamicin (RAFA Labs, injectable grade), and 0.1% amphotericin B (BMS, Fungizone I.V.).

Microdermis production. Following harvesting, each dermal core was transferred to a new well containing 1 ml of DMEM production medium and then washed twice more. The microdermises were equilibrated in 1 ml of medium at 37°C, 5% CO_2 for a 24-h recovery period. An aliquot of Ad5-CMV-hEPO or Ad5-LacZ was thawed at room temperature and diluted in DMEM production medium, without serum, to 10^{11} VP/ml. The medium surrounding each microdermis was carefully removed, and then each core was transduced with 250 μl of diluted virus at 37°C, 5% CO_2 for 24 h, with shaking (300 rpm) during the initial 2 h. After 24 h the transduction medium was removed and the microdermises were washed six times with 2 ml of DMEM production medium and then returned to the incubator in 1 ml of DMEM production medium. The medium was changed every 3 days. Microdermises transduced with AAV1/2-hEPO (1.1×10^{11} VP/ml) were washed and maintained in production medium as described above for the adeno vectors. The production medium from days 5 and 8 after harvest was analyzed for glucose consumption (Glucose (GO) Assay Kit GAGO 20; Sigma) and for hEPO concentration and secretion levels by ELISA (Quantikine human erythropoietin; R&D Systems, Minneapolis, MN, USA), according to the manufacturers' instructions.

Biochemical analysis of secreted hEPO. The medium was collected from wells containing microdermal hEPO and concentrated by filtration. Western blots of microdermis culture medium, containing 25 ng of hEPO or BRP recombinant hEPO (European Pharmacopoeia Commission Cat. No. E1515000), were performed with a hEPO specific monoclonal antibody (MAB 2871, 1:500; R&D Systems). The samples were also applied to a reverse-phase chromatography column (214TP54 C-4, 5¼ m; Vydac) and a protein fraction corresponding to a standard (BRP) of rhEPO was recovered. The purified protein was used for N′-terminal sequencing and mass spectrometry analysis. For N′-terminal sequencing, 1.0 μg of the protein was sequenced by an automated Edman sequencer (Procise 494HT; Applied Biosystems, Foster City, CA, USA). For mass spectrometry 1.0 μg of the protein was digested with trypsin and separated by reverse-phase HPLC. The eluted peptides were analyzed using the electrospray ionization method. The peptides were fragmented in the mass spectrometer (electrospray ion trap mass spectrometer; LCQ Classic ThermoFinnigan, San Jose, CA, USA) and the molecular masses of the resulting peptides were determined.

Immunohistochemistry of the microdermis. To analyze the efficiency of transduction, and to ascertain which cells of the dermal structure are transduced by Ad5-CMV-hEPO, microdermises were fixed in 10% buffered paraformaldehyde and 4- to 5-μm paraffin-embedded sections were prepared for staining with H&E or anti-hEPO or anti-vimentin monoclonal antibodies (R&D Systems).

Implantation of microdermis into SCID mice. All SCID mouse procedures were performed at a GLP-certified site (Harlan Biotech, Rehovot, Israel) and approved by the national Small-Animal Ethics Committee. Briefly, the microdermis was loaded into a 14-gauge hypodermic needle (Air-Tight, VA, USA) by suction. The needle was run under the subcutaneous space and while retracted, the microdermis was gently placed under the dermis. Hematocrit levels in the mouse blood were determined by the standard capillary method.

Toxicology. SCID mice (both males and females) were implanted subcutaneously (dorsal implantation) with human EPO microdermis on

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day 8 after skin harvest. Fourteen mice were implanted with one microdermis/mouse, 14 mice with two microdermises/mice, and an additional control group with microdermis transduced with Ad/null. Termination of the study and toxicology analysis was performed 28 days after implantation, by a specialist veterinarian and a histopathologist. They examined the implantation site and the following organs: lymph nodes, heart, liver, kidney, spleen, and bone marrow. It should be noted that in this study the mice were subjected to hEPO levels that are hundreds of times higher than the dosages planned for humans. The findings were that the treatment "did not cause any major adverse toxic effects." (Studies were carried out under GLP procedures by The Harlan Laboratories Ltd., Rehovot, Israel.)

Statistics. All *in vitro* and *in vivo* experiments were carried out in replicate samples and results are presented as averages (standard deviation).

The r^2 was calculated according to Pearson, which returns the square of the Pearson product moment correlation coefficient through data points in known $y's$ and known $x's$.

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REFERENCES

1. Winearls, C. G. (1995). Historical review on the use of recombinant human erythropoietin in chronic renal failure. *Nephrol. Dial. Transplant.* **10:** 3–9.
2. Besarab, A. (2000). Physiological and pharmacodynamic considerations for route of EPO administration. *Semin. Nephrol.* **20:** 364–374.
3. Schagen, F. H., Ossevoor, M., Toes, R. E., and Hoeben, R. C. (2004). Immune responses against adenoviral vectors and their transgene products: a review of strategies for evasion. *Crit. Rev. Oncol. Hematol.* **50:** 51–70.
4. Chang, P. L. (1999). Encapsulation for somatic gene therapy. *Ann. N. Y. Acad. Sci.* **875:** 146–158.
5. Grad-Itach, E., *et al.* (2003). Liver micro-organs transcribe albumin and clotting factors and increase survival of 92% hepatectomized rats. *J. Hepatol.* **39:** 552–558.
6. (Freinkel, R. K., Woodley, D. T. Eds.). Biology of the Skin (2001). Parthenon Pub. Group, London.
7. Lopez-Soto-Yarritu, P., Diez-Masa, J. C., Cifuentes, A.., and de Frutos, M. (2002). Improved capillary isoelectric focusing method for recombinant erythropoietin analysis. *J. Chromatogr.* **968:** 221–228.
8. Baek, S. C., Lin, Q., Robbins, P. B., Fan, H., and Khavari, P. A. (2001). Sustainable systemic delivery via a single injection of lentivirus into human skin tissue. *Hum. Gene Ther.* **12:** 1551–1558.
9. Siprashvili, Z., and Khavari, P. A. (2004). Lentivectors for regulated and reversible cutaneous gene delivery. *Mol. Ther.* **9:** 93–100.
10. Dalle, B., *et al.* (1999). Improvement of mouse beta-thalassemia upon erythropoietin delivery by encapsulated myoblasts. *Gene Ther.* **6:** 157–161.
11. Sommer, B., *et al.* (2002). Long-term doxycycline-regulated secretion of erythropoietin by encapsulated myoblasts. *Mol. Ther.* **6:** 155–161.
12. Schwenter, F., Schneider, B. L., Pralong, W. F., Deglon, N., and Aebischer, P. (2004). Survival of encapsulated human primary fibroblasts and erythropoietin expression under xenogeneic conditions. *Hum. Gene Ther.* **15:** 669–680.
13. Palmer, T. D., Rosman, G. J., Osborne, W. R., and Miller, A. D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. *Proc. Natl. Acad. Sci. USA* **88:** 1330–1334.
14. Scharfmann, R., Axelrod, J. H., and Verma, I. M. (1991). Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc. Natl. Acad. Sci. USA* **88:** 4626–4630.
15. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997). Construction of adenovirus vectors through Cre–lox recombination. *J. Virol.* **71:** 1842–1849.
16. Hauck, B., Chen, L., and Xiao, W. (2003). Generation and characterization of chimeric recombinant AAV vectors. *Mol. Ther.* **7:** 419–425.