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Letter to the Editor

Scar trek: follicular frontiers in skin replacement therapy

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This is the first report on *in vivo* epithelial stem cell (ESC) transfection using intradermal injections of genes encapsulated in liposomes. Skin gene therapy is a relatively new approach with great potential because of the accessibility and ease with which the modified area can be monitored (Alexander and Akhurst, 1995). Liposomes have become attractive as gene therapy vectors due to their non-viral composition, stability, and ability to interact with the cell membrane (Felgner, 1996). We have successfully utilized cationic liposomes as efficient vectors to deliver genes for growth factors such as insulin-like growth factor-I (Jeschke et al., 2000) and keratinocyte growth factor (Jeschke et al., 2002), in a previously described murine model of thermal injury (Jeschke et al., 2001).

Liposomes were formulated with 1:1 (M/M) 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyl ethyl ammonium bromide and cholesterol suspended in membrane-filtered water (Life Technologies, Rockville, MD, USA). *LacZ* cDNA encoding for the non-mammalian enzyme, β-galactosidase, was used to confirm transfection. The presence of the β-galactosidase protein was detected by histochemical staining with Bluo-Gal (halogenated indolyl- β-D-galactosidase) (Life Technologies, Gaithersburg, MD, USA) (Jeschke et al., 1999). We noticed positive transfection of hair follicle cells at the wound edge (i.e., at the site of intradermal injections) in these experiments (Figure 1A,B). In order to see if these Bluo-Gal-positive cells were indeed ESCs, we dual-stained these sections with anti-cytokeratin 19 (CK19) antibodies (Abcam Inc., Cambridge, MA, USA) using standard immunohistochemical techniques. CK19 is a known marker for hair follicle stem cells (Michel et al., 1996; Brembeck and Rustgi, 2000). As shown in Figures 2 and 3, CK19 was co-localized on Bluo-Gal-positive cells, indicating successful transfection of ESC.

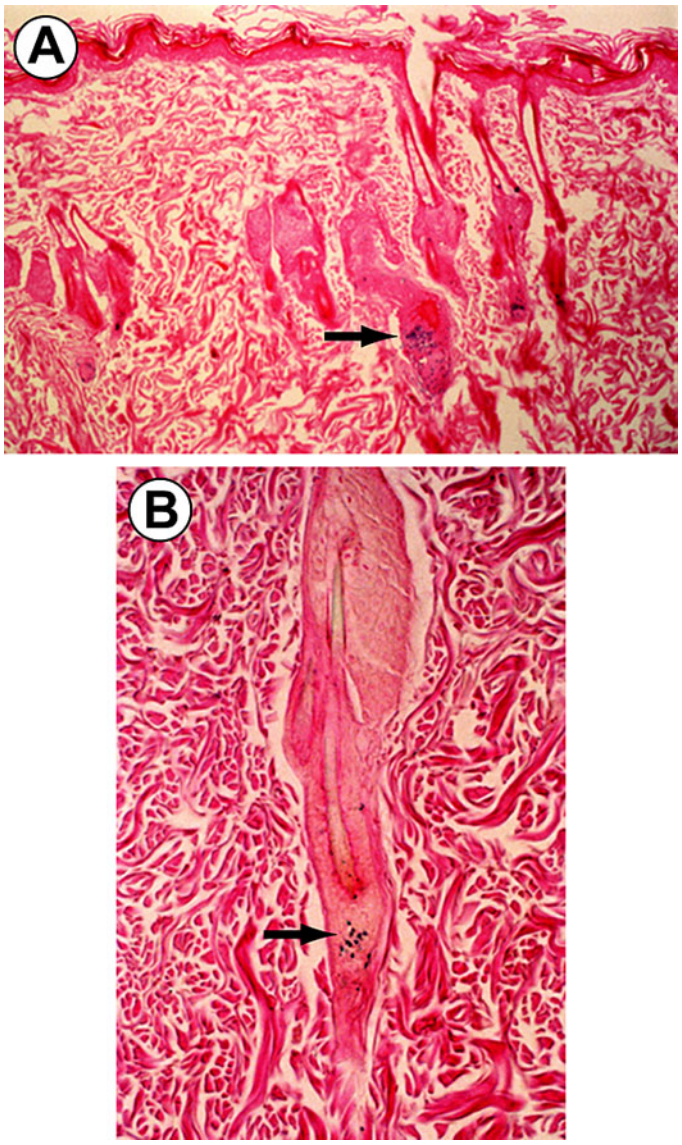

Figure 1. Four-micron sections stained for β-galactosidase and counterstained with eosin. **A.** Magnification = 200X; **B.** magnification = 400X. Hair follicle cells (marked with arrows) show positive reaction for β-galactosidase (blue color) and thus *lacZ* transfected.

This is the first report on transfection of ESCs using intradermal injections of liposomes. The feasibility of using topical applications of liposomes has been previously reported both *in vitro* and *in vivo* for both murine and human hair follicles (Jones et al., 2002; Davey, 2003). Domashenko et al. (2000) showed efficient transfection of human follicle cells after topical application of liposome-entrapped *lacZ* in mice as well as in a human scalp xenograft model. Normally, the hair follicle continuously cycles through three major stages: anagen - the hair

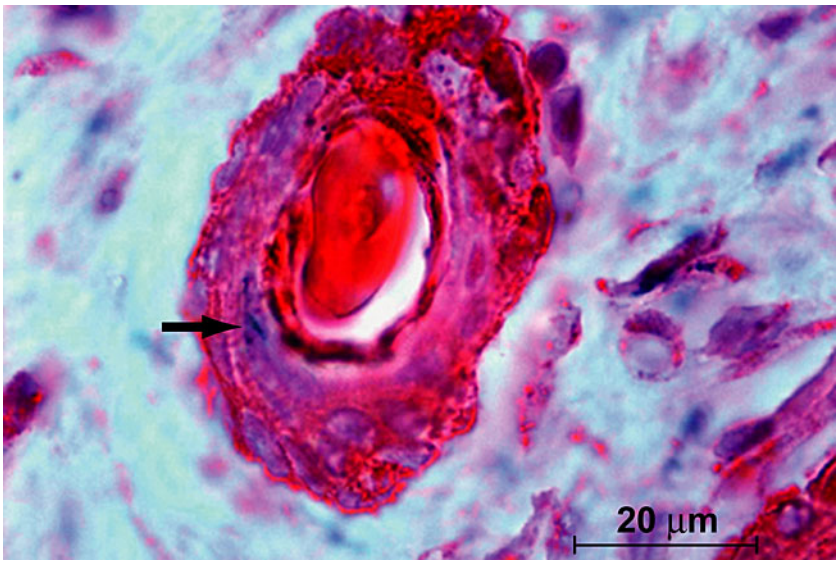

Figure 2. Four-micron sections stained for β-galactosidase (blue color) and dual stained with anti-cytokeratin-19 (CK-19) antibodies using peroxidase immunohistochemical technique (magnification = 1000X). Alkaline phosphatase was used as substrate, and hence, bright pink-stained cells are CK-19 positive. Sections were lightly counterstained with hematoxylin (5 s) to identify nuclei. Section shows co-localization of CK-19 and Bluo-Gal stain in hair follicle cells (epithelial stem cell, arrow).

growth phase, catagen - the involution phase, and telogen - the resting phase. Domashenko et al. (2000) found that liposome composition, timing of liposome application to the onset of a new hair cycle, and pretreatment with depilation and retinoic acid were important for transfection efficiency. Pretreating the skin with depilation and/or retinoic acid reverted the follicles to the beginning of anagen. Transfection rates were high in our study, since many follicles at the site of injection were positively stained. We did not pretreat our skin in any way prior to transfection; however, since trauma to or adjacent to hair follicles can revert the cycle to anagen phase, this may explain why the follicles adjacent to the wound were mostly transfected. Also, similar to findings by Domashenko et al. (2000), β-galactosidase activity was detected 5 days after the last injection. Thus, intradermal injection of lipoplexes had a similar length of activity and area of activity as topical application. However, in studies where lipoplexes were topically applied, no other cells were transfected with *lacZ* outside the follicle in the epidermis or dermis (Jones et al., 2002; Davey, 2003). This demonstrates that genes can be selectively targeted to ESCs of the hair follicle. Contrary to this finding, intradermal injection of lipoplexes in our study seemed to transfect dermal sheath cells, in addition to follicle matrix cells (Figure 3). This is important, since dermal sheath cells have a degree of immune privilege, allowing for allogenic transplant without rejection. They have also been implicated in regulating the cellularity and architecture of the dermis (Chen et al., 2002). The dermal-epidermal tissue interactions that underlie skin development persist in mature hair follicles. Dermal sheath cells play a prominent part in the developmental activity and remodeling at this site (Chen et al., 2002). Hence, a greater appreciation of dermal sheath cell function and their modulation through intradermal transfection is of clinical relevance.

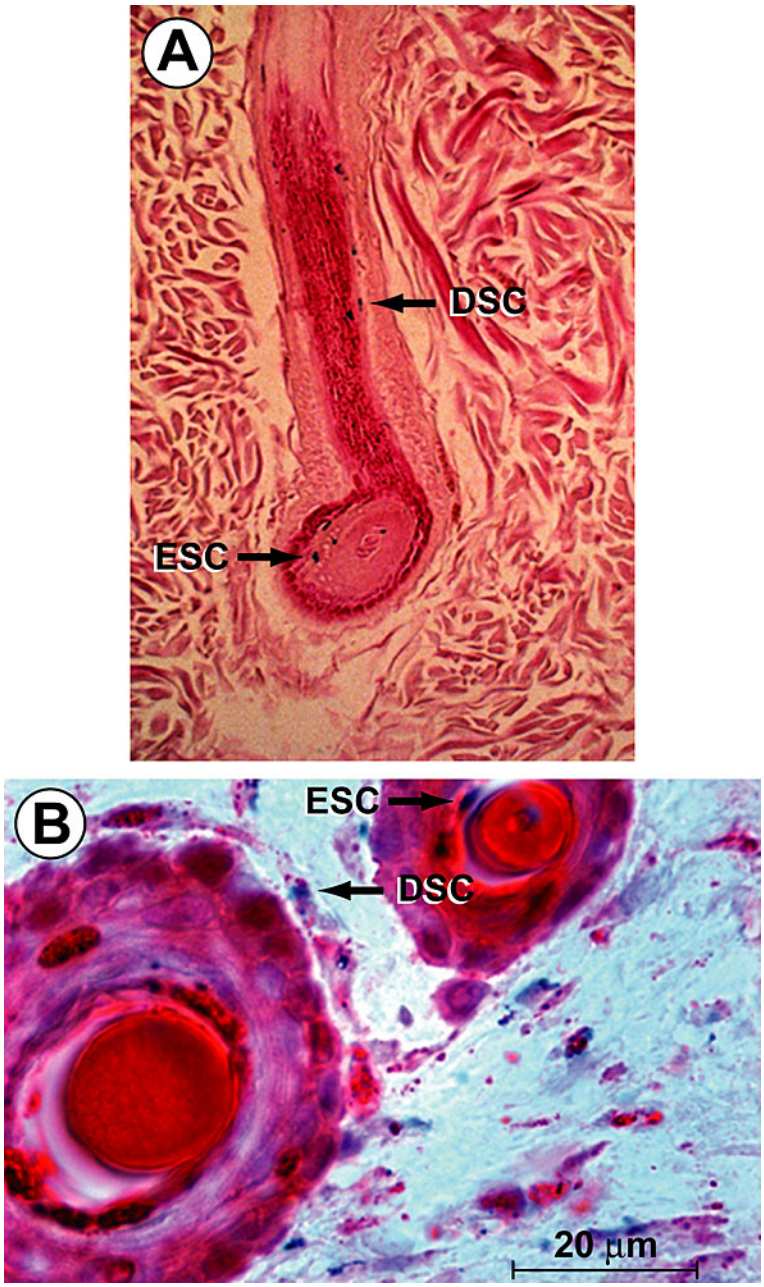

Figure 3. Four-micron sections stained for β-galactosidase (blue) (**A**) and dual stained with anti-cytokeratin-19 (CK-19) antibodies using peroxidase immunohistochemical technique (magnification = 1000X) (**B**). Alkaline phosphatase was used as substrate, and hence, bright pink-stained cells are CK-19 positive. Sections were lightly counterstained with hematoxylin (5 s) to identify nuclei. Section shows co-localization of CK-19 and Bluo-Gal stain in dermal sheath cells (DSC) along with hair follicle cells (epithelial stem cell, ESC).

Also, *ex vivo* approaches allow close to 100% gene transfer (Brigham and McLoughlin, 1996; Rheinwald and Green, 1975) and can be applied to the hair follicle. This takes on added

importance in light of the recent discovery that hair follicle dermal sheath cells taken from the scalp of an adult human male could form new dermal papilli and hair follicles that produced hair shafts when transplanted in the skin of an unrelated female (Brigham and McLoughlin, 1996). This type of transplantation takes advantage of the so-called immune privilege of hair follicle cells that prevents their rejection, and the possible totipotency or inductive properties of the follicle dermal sheath cells (Brigham and McLoughlin, 1996). This gives rise to many possibilities for genetically altering hair follicles *ex vivo* for subsequent reimplantation or even transplantation to individuals other than the donor.

The above mentioned points have thrilling clinical implications, considering the regenerative and proliferative potential of ESCs and the ease with which they are cultured (Singer and Clark, 1999). Loss of integrity of the skin can lead to major disability or even death. In the United States alone, over 2 million people suffer from burns each year (Taylor et al., 2000). Major burns result in extensive full thickness skin defects, often leading to scar contractures, altered thermoregulation, and unsatisfactory cosmetic results (Taylor et al., 2000). Current standards of burn care involve early excision of burn wounds followed by prompt closure. This has led to the routine survival of burn injuries involving more than 80-90% of the total body surface area. Paucity of donor sites for coverage, in these cases however, has led to the use of 'skin substitutes' that utilize a combination of dermal fibroblasts (usually from neonatal foreskin) incorporated into biodegradable synthetic and allogenic matrix structures. This allows the cells to synthesize the extracellular matrix, thus reconstructing the dermal component of skin (Oshima et al., 2001). These are usually covered with thin autologous epithelium, which serves to replace the epidermal component of skin, allowing the possibility of mimicking normal skin. In addition, cultivated epithelial autograft is now a well-established and indeed a life-saving procedure that uses sheets of the patient's own epithelial cells to cover large defects. However, thin autografts and cultivated epithelial autograft are essentially composed of fully differentiated keratinocytes that are fragile and prone to repeated breakdown (Rochat et al., 1994). Although many of the skin substitutes attempt to rebuild the dermis to resemble normal skin, not much work has been done on reconstructing the epidermis to resemble normal epidermis. The use of ESCs has exciting possibilities in this area. Multipotent stem cells isolated from adult hair follicles have the capacity to reconstitute a wounded epidermis (Domashenko et al., 2000), and respond to skin morphogenetic signals by forming epidermis, hair follicles and sebaceous glands (Li and Hoffman, 1995). In fact, a single adult skin stem cell has sufficient proliferative capacity to produce enough new epidermis to cover the body surface (Brigham and McLoughlin, 1996). The concept is to use the embryonic-like and interactive properties of hair follicle cells to engineer a new generation of composite skin substitutes, which will grow new hair follicles and other skin appendages when grafted. Further, 7 million people suffer from chronic wounds caused by diabetes, pressure, venous diseases, and arterial diseases in the United States each year (Bittira et al., 2002). Skin substitutes would also be important in such cases. Other skin disorders such as dystrophic epidermolysis bullosa and lamellar ichthyosis are also potential candidates for ESC gene therapy (Choate et al., 1996; Reynolds et al., 1999). Cultured ESCs from patients with dystrophic epidermolysis bullosa, a disease characterized by skin blisters and caused by mutations in the COL7A1 gene (which encodes for collagen VII), were corrected by genomic integration and transplanted into immunodeficient mice (Reynolds et al., 1999). The transplanted cells maintained the corrected expression through multiple cell turnovers (Choate et al., 1996; Reynolds et al., 1999). These studies present possible *ex vivo* strategies to correct skin disor-

ders caused by genetic lesions through non-viral and lentiviral approaches. However, long-term correction must be ensured before such methods are applied in the clinic.

During the last few years, somatic stem cell biology has become an area of great importance. On the one hand, it has contributed to the study of basic mechanisms involved in cell proliferation and differentiation; on the other hand, it has proven essential for the development of cellular therapy, an emerging biomedical field of significant relevance in the treatment of several disorders. It is evident that the plasticity shown by different types of somatic stem cells both *in vitro* and *in vivo* will have clinical applicability in the years to come. Understanding first the nature and qualities of tissue-specific stem cells and second the mechanisms by which tissue-specific stem cells differentiate into mature, functional cells is essential if we are to manipulate them safely. We need to know more about the intrinsic molecular controls that keep stem cells pluripotent or direct them along particular differentiation pathways. Such intrinsic regulators are, in turn, sensitive to the influences of the microenvironment. For example, marrow stromal stem cells have recently been shown to undergo milieu-dependent differentiation and to express phenotypes similar to their surrounding cells. *In vitro* preprogramming of these cells can be used to augment myocardial angiogenesis and myogenesis after myocardial infarction in rats (Bittira et al., 2002). The spotlight on stem cells has revealed gaps in our knowledge that must be filled if we are to take advantage of their full potential for treating devastating injuries such as severe burns. We compliment Dr. Morris and her colleagues for their exciting work and thank the editor for the opportunity to share our findings and perspectives in this area.

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