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## Healing Cells in the Dermis and Adipose Tissues of the Adult Pig

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### ABSTRACT

Stage-specific antigen-4 (SSEA-4) positive cells and carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) positive cells, indicative of pluripotent stem cells and totipotent stem cells, respectively, have been isolated and characterized from the skeletal muscle and blood of adult animals, including humans. The current study was undertaken to determine their location in the dermis and underlying connective tissues of the adult pig. Adult pigs were euthanized following the guidelines of Fort Valley State University's IACUC. The skin (epidermis through hypodermis) was harvested, fixed, cryosectioned, and stained with the two antibodies: SSEA-4 and CEA-CAM-1. SSEA-4 positive cells were located preferentially in the reticular dermis of the skin and to some extent in the underlying hypodermis. In contrast, CEA-CAM-1 positive stem cells were preferentially located within the hypodermis of the pig skin within the loose fibrous connective tissues surrounding adipose tissue. CEA-CAM-1 positive cells were also located, to a lesser extent, in the dermis as well. These results demonstrate the presence of native populations of pluripotent stem cells and totipotent stem cells within the dermis, hypodermis, and adipose tissue of adult pig skin. Studies are ongoing to address the functional significance of these cells in normal injury and repair.

### Keywords

Healing cells, Totipotent stem cells, Pluripotent stem cells, SSEA, CEA-CAM-1, Adult pig, Porcine, Dermis, Fat, Skin, Adipose tissue, Immunocytochemistry, ELICA-fixative.

### Introduction

There are three basic categories of cells within animals, i.e.,

functional cells, maintenance cells, and healing cells. The functional cells comprise the majority of the cell types and are composed of both stroma and parenchyma. They interact on a day-to-day basis with the animal's external and internal environments [1]. A few examples are of functional cells are adipocytes, fibrocytes, and myocytes. Maintenance cells support the functional cells on a daily basis by replacing functional cells as they wear

out and die as well as providing trophic factors for their function and survival. A few examples of maintenance cells are adipoblasts, fibroblasts, myoblasts, mesenchymal stem cells, medicinal secreting cells, and progenitor cells [1-6]. Healing cells are normally dormant and can be found hibernating within the stromal connective tissues throughout the body [7,8]. Their function is to replace functional cells and maintenance cells lost due to trauma and/or disease. Examples of healing cells are totipotent stem cells [1,6,8,9], pluripotent stem cells [1,6,10-12], ectodermal stem cells [1,6], mesodermal stem cells [1,6,13], and endodermal stem cells [1,6]. Healing cells comprise approximately 10% of all the cells of the body. They are ubiquitous, as they are found throughout all organs and tissues of the body. More specifically, totipotent stem cells comprise approximately 0.1%, pluripotent stem cells approximately 0.9%, and the ectodermal stem cells, mesodermal stem cells, and endodermal stem cells taken together, approximately 9% of all cells of the body [1,6,14].

The location, characterization, and activities of endogenous healing cells have been studied in skeletal muscle [2,8], bone marrow [15-20], blood [10,21], adipose tissue [22], and at least 33 other tissues and organs within the body in multiple species of animals. Animals examined thus far include amphibians (Ambystoma Salamanders), reptiles (Komodo Dragon), avians (chicken, Waddell Crane), and 12 species of mammals, including humans (i.e., mice, rats, rabbits, dogs, cats, sheep, goats, pigs, cows, Speckled Bear, horses, and humans) [1,6,7,10,23-29]. Healing cells have also been found in human umbilical cord, amnion, and placenta [30-32].

Native healing stem cells are located in the bone marrow (20), skeletal muscle (8), and blood stream (10,21) of various animal species. The current study was therefore designed to determine the location of healing cells in the skin of the adult pig. Discovery of these healing cells could provide an important initial step toward the ultimate goal of successful and safe cellular therapy for the treatment of a wide variety of conditions involving damage to the skin.

## Materials and Methods

### Animal Use

The use of animals in this study complied with the guidelines of the Institutional Animal Care and Use Committee of Fort Valley State University and with the criteria of the National Research Council for the humane care of laboratory animals as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Academy Press, 1996).

### Tissue Harvest

Twenty adult 120 lb. female Yorkshire pigs (n=20) were anesthetized with tiletamine and zolazepam, and then prepared for surgery with a Betadine wash. Sterile drapes were placed, and one-inch wide skin slices were made on either side of a midline laparotomy incision. The slices included the epidermis, dermis, and hypodermis with embedded adipose tissue. The skin was sliced into one-inch square pieces and placed in 500-ml wide-

mouth tissue culture jars (Corning, NY) containing 400-ml of cold ELICA fixative. The ELICA fixative consisted of aqueous 0.4% v/v glutaraldehyde, 2% w/v paraformaldehyde, and 1% w/v glucose, Ph 7.4, with an osmolality 1.0 [8]. The porcine skin was allowed to remain in the fixative for 1 to 24 weeks at ambient temperature. After fixation, the skin was transferred and stored in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen, GIBCO, Grand Island, NY) at pH 7.4 and ambient temperature. Pieces of skin and associated adipose tissue were removed, placed into Tissue Tek OCT Compound 4583 (Miles Laboratory, Ames Division, Elkhart, IN) and then frozen at -20°C. The frozen pieces of skin were cryostat sectioned at seven microns in thickness with a Tissue Tek Cryostat II (GMI, Ramsey, MN), placed on positively charged slides (Mercedes Medical, Sarasota, FL) and refrigerated at -20°C. Immunocytochemical staining was performed following established procedures for ELICA analysis [8,33].

### Immunocytochemistry

Seven-micron tissue sections were incubated with 95% ethanol to remove the OTC cryostat embedding medium and then washed under running water for five minutes. The tissue sections were incubated with 5.0% (w/v) sodium azide (Sigma, St. Louis, MO) in DPBS for 60 minutes. They were then washed in running water for five minutes, and incubated with 30% hydrogen peroxide (Sigma, St. Louis, MO) for 60 minutes to irreversibly inhibit endogenous peroxidases [34]. Tissue sections were rinsed with running water for five minutes and incubated for 60 minutes with blocking agent (Vecstatin ABC Reagent Kit, Vector Laboratories Inc., Burlingame, CA) in DPBS [33]. The blocking agent was removed and the sections rinsed with running water for five minutes. They were then incubated with primary antibody for 60 minutes. The primary antibodies consisted of 0.005% (v/v) carcinoembryonic antigen cell adhesion molecule-1 (CEA-CAM-1) in DPBS for totipotent stem cells [9]; 1 µg per ml of stage-specific embryonic antigen-4 for pluripotent stem cells (SSEA-4, Developmental Studies Hybridoma Bank, Iowa City, IA) in DPBS [6,12]; and smooth muscle alpha-actin (IA4, Developmental Studies Hybridoma Bank) in DPBS [8,20]. The primary antibody was removed. The sections were rinsed with running water for five minutes, and incubated with secondary antibody for 60 minutes. The secondary antibody consisted of 0.005% (v/v) biotinylated affinity-purified, rat adsorbed anti-mouse immunoglobulin G (H + L) (BA-2001, Vector Laboratories) in DPBS [8]. The secondary antibody was removed. The sections were rinsed with running water for five minutes, and then incubated with avidin-HRP for 60 minutes. The avidin-HRP consisted of 10 ml of 0.1% (v/v) Tween-20 (ChemPure, Curtin Matheson Scientific, Houston, TX) containing 2 drops reagent-A and 2 drops reagent-B (Peroxidase Standard PK-4000 Vectastain ABC Reagent Kit, Vector Laboratories) in DPBS [8]. The avidin-HRP was removed. The sections were rinsed with running water for five minutes, and incubated with AEC substrate (Sigma) for 60 minutes. The AEC substrate was prepared as directed by the manufacturer. The substrate solution was removed. The sections were rinsed with running water for 10 minutes and then covered with Aqua-mount (Vector Laboratories) [8].

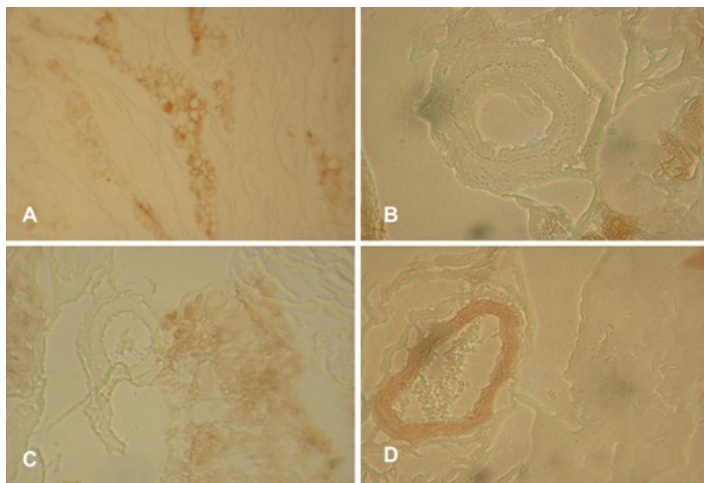
Positive and negative controls were included to assure the validity of the immunocytochemical staining [8]. The positive controls consisted of adult-derived totipotent stem cells (positive for CEA-CAM-1) [8,9], pluripotent stem cells (positive for SSEA-4) [6,12], and smooth muscle surrounding blood vessels within the tissue (positive for IA4) [8,20]. The negative controls consisted of the staining protocol with DPBS alone (no antibodies or substrate), without primary antibodies (CEA-CAM-1, SSEA-4, or IA4), without secondary antibody (biotinylated anti-mouse IgG), without avidin-HRP, and without substrate (AEC) [8].

### Visual Analysis

Stained sections were visualized using a Nikon TMS phase contrast microscope with bright field microscopy at 40x, 100x, and 200x. Photographs were taken with a Nikon CoolPix 995 digital camera.

### Results

Cells that exhibited positive staining for CEA-CAM-1 were located in the loose fibrous connective tissue surrounding the adipose tissue in the hypodermis of adult porcine skin (Figure 1A), as well as in the fibrous connective tissue surrounding the blood vessels within the reticular layer of the dermis (Figure 1B). Cells that exhibited positive staining for SSEA-4 were located preferentially within the fibrous connective tissue of the reticular layer of the dermis of adult porcine skin (Figure 1C). Positive staining for smooth muscle alpha-actin was used as a positive procedural control. Such staining was apparent within the tunica media of a blood vessel located in the reticular layer of the dermis (Figure 1D). Negative procedural controls demonstrated absence of staining within the dermis or hypodermis of porcine skin (data not shown).



**Figure 1:** Cryosectioned porcine dermis and hypodermis. **A:** CEA-CAM-1-positive cells located within loose fibrous connective tissue stroma surrounding unilocular white fat cells in hypodermis of porcine skin, 40x mag. **B:** CEA-CAM-1-positive cells located within the fibrous connective tissue stroma surrounding an unstained arterial blood vessel within the reticular layer of the dermis, 200x mag. **C:** SSEA-4-positive cells located within the fibrous connective tissue stroma in the border region between the reticular layer of the dermis and the hypodermis, 100x mag. **D:** IA4-positive cells (positive procedural control for smooth muscle

alpha-actin) within the tunica media of a vein within reticular layer of dermis. Note the presence of unstained red blood cells within the lumen of the vein. 200x mag.

### Discussion

Young et al. previously reported the presence of totipotent stem cells (TSCs) and pluripotent stem cells (PSCs) in a variety of species of post-natal mammals [9,14,35]. Both stem cells have shown the capacity for extended self-renewal and the potential to form any somatic cell of the body. Stout et al. [28] demonstrated that these stem cells circulate in the peripheral blood as well as reside within the skeletal muscle of adult pigs. These cells were liberated from the skeletal muscle into the peripheral blood after only 90 minutes of trauma [28]. These findings led to the current study which identified and located CEA-CAM-1-positive totipotent stem cells and SSEA-4-positive pluripotent stem cells in adult porcine skin. Further studies are necessary to further characterize and utilize these cells in both veterinary and human therapeutic applications.

Clonal populations of totipotent stem cells and pluripotent stem cells have been derived by repetitive serial dilution clonogenic analysis in the adult rat [9,12]. These cells can differentiate to form cells belonging to all three germ layer lineages, i.e., ectoderm, mesoderm, and endoderm [6]. For example, rat brains were lesioned with 6-hydroxydopamine as a model of Parkinson disease. After stereotactic injection of Lac-Z-transfected undifferentiated pluripotent stem cells, the genomically-labeled stem cells formed dopaminergic neurons, cortical pyramidal neurons, interneurons, glial cells, and vasculature [36]. Another example involves lesioning of a coronary artery as a model of myocardial infarction. Cultured Lac-Z-transfected undifferentiated pluripotent stem cells were injected either directly into the area of the lesion or administered systemically by intravenous injection. These genomically-labeled cells incorporated into the myocardium, demonstrating a possible role in the restoration of the histoarchitecture of the damaged myocardium [12]. These same cells have also been shown to generate three-dimensional pancreatic islet-like structures that responded to a glucose challenge by secretion of species-specific insulin [37]. Taken together, these results suggest that the regeneration and repair of one organ or tissue may be accomplished by totipotent stem cells and pluripotent stem cells residing in another organ or tissue.

Much of the research on therapeutic approaches to the treatment of inherited skin disorders, such as xeroderma pigmentosum, involves the use of gene therapy employing a viral vector [38]. Young et al. proposed that pluripotent stem cells could provide a means for delivery of gene therapy without loss of the development potential of the stem cells themselves [14]. Thus, based on the Lac-Z genomically-labeling studies [12,36], a transfected pluripotent stem cell could serve as a vector for gene therapy while simultaneously providing a source of cells with normal tissue function.

Skin ages as a consequence of decreased cutaneous elasticity and resilience [39]. The development of wrinkled skin is associated

with increased susceptibility of elastic fibers in the dermal extracellular matrix to proteolytic degradation. These changes are a consequence of the inability of adult dermal fibroblasts to synthesize elastin to replace the degraded elastic fibers. Healing cells (in the form of totipotent stem cells, pluripotent stem cells, and/or mesodermal stem cells), could provide a source of functional pre-fibroblasts or cells that provide fibroblasts with the appropriate signals required for their survival. In addition to their possible uses for cosmetic purposes in the treatment of aging in skin, dermal stem cells could provide a possible treatment option for inherited diseases that impair the deposition of elastic fibers.

Ectodermal stem cells are known to exist in the bulge of the hair follicle. Such stem cells are capable of regenerating the hair follicle and epidermis in response to burns or other types of wounds [40]. Stem cells in the follicular bulge are damaged in certain types of alopecia, particularly those involving inflammation and permanent loss of follicles [41]. Healing cells, such as totipotent stem cells and pluripotent stem cells, isolated from various tissue locations such as the dermis [this study,7], adipose tissues [this study,22], skeletal muscle [7,8,25,28], blood [10,21,28,29], or bone marrow [11,17,19,20], may provide a source of functional stem cells for repopulating the follicular bulge to facilitate therapeutic regeneration of hair.

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