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Skin-derived precursors generate enteric-type neurons in aganglionic jejunum

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ABSTRACT

Purpose: Skin-derived precursor cells (SKPs) may regenerate the enteric nervous system in Hirschsprung's disease. SKPs migrate and differentiate into myenteric ganglia in aganglionic intestine. We sought to characterize the time-course of SKP gangliogenesis and enteric neurotransmitter synthesis *in vivo*.
Methods: Adult Lewis rat jejunal segments were isolated and denervated with benzalkonium chloride (BAC). Denervation was evaluated by immunohistochemical (IHC) stains for markers of mature neuronal and glial cells. Green fluorescent protein (GFP)-expressing neonatal rat SKPs were cultured in neuroglial-selective medium. SKPs were transplanted into aganglionic segments 65–85 days after BAC treatment. IHC was performed to identify glia, neurons, and neurotransmitter synthesis in GFP + cells between post-transplant days 1 and 28.
Results: Aganglionosis was confirmed by IHC. On post-transplant days 1 and 2, GFP + cells were detected near injection sites within the muscularis propria. GFP + cell clusters were evident only between longitudinal and circular smooth muscle layers at post-transplant days 14, 21, and 28. These structures co-expressed markers of mature neurons and gliocytes. Several markers of neurotransmitter synthesis were detected in GFP + clusters at days 21 and 28.
Conclusion: SKPs are capable of enteric neuroglial differentiation *in vivo*. SKPs migrate to the intermuscular layer of aganglionic intestine within days of transplantation. Our observations suggest that SKPs are capable of generating enteric ganglia in aganglionic intestine.

Gastrointestinal neuromuscular dysfunction (GND) is characterized by the absence of functional enteric ganglia, resulting in dysmotility of gastrointestinal smooth muscle. It is a significant source of morbidity and mortality for millions of people worldwide, incurring an estimated global cost burden of over $600 million per year [1,2]. GND typifies several disorders, including Hirschsprung's disease, diabetic gastroparesis, Chagas disease, esophageal achalasia, visceral neuropathies, and ganglioneuromatosis [3–7]. In all of these conditions, the neurologic insult is irreversible.

Pharmacological stimulant therapies for GND produce little benefit, and surgical options for most of these conditions are severely limited. In general, the outcomes of available therapies are unfavorable, and patients often suffer debilitating chronic pain or nutritional deficiency [8,9]. For patients with esophageal achalasia or Hirschsprung's disease, surgical correction is potentially curative; however, healthcare costs associated with these conditions are substantial, and the potential complications of operative interventions include bleeding, infection, chronic inflammation, perforation, sepsis, and death [3,6,9–15]. Cell-based therapy offers a potentially regenerative alternative to existing treatment modalities.

Several investigators have reported successful engraftment of neural crest-derived stem cells within aganglionic segments of the gastrointestinal tract [16–20]. Skin-derived precursor cells (SKPs) are a subpopulation of neural crest-derived cells that reside in the perivascular dermis layer of the skin [21,22]. Clonal characterization analyses have shown that SKPs maintain multipotency and persist into adulthood [23–26]. We recently discovered that SKPs are capable of gangliogenesis within aganglionic jejunum *in vivo* [27]. Nevertheless, the physiologic function of neurons in these SKP-derived ganglia is indistinct. Kwok and colleagues have shown that SKPs generate neurons capable of enteric neurotransmitter synthesis within intestinal explants [28]. In this study, we present the cellular physiological characteristics and time course of gangliogenesis achieved by SKP transplantation *in vivo*.

1. Materials and methods

1.1. Animal subjects

The following was conducted with the approval of our Institutional Animal Care and Use Committee under protocol #2006-061. Adult female Lewis rats (n = 9) were obtained from Charles River Laboratories (Wilmington, MA). Neonatal Lewis rat pups at day of life 3 (n = 6) were

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used for skin cell isolation. The rat pups ubiquitously expressed enhanced green fluorescent protein (GFP) for later cell identification on fluorescence microscopy [29]. Animals were boarded in United States Department of Agriculture-approved housing conditions with veterinary surveillance for the duration of this study.

1.2. SKP isolation and culture

Neonatal rat GFP expression was confirmed by ultraviolet microscopic visualization of tail clippings from euthanized pups (excitation $\lambda = 490$ nm). SKPs were harvested following the protocol described by Biernaskie and colleagues [25,27]. For each SKP isolation, skin specimens were obtained from 2 pups for optimal yield. SKP isolations were performed among 3 litters, and cell yields were quantified with a hemacytometer. SKPs were plated at a density of 5.0×10^5 cells per T75 flask in NeuroCult® NS-A Basal Medium (Rat) with 10% Proliferation Supplement, 0.0002% heparin solution (StemCell Technologies, Vancouver, Canada), 1× Antibiotic Antimycotic (ABAM) solution (Invitrogen, Carlsbad, CA), 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), and 20 ng/ml basic fibroblast growth factor (bFGF) (Peprotech). SKPs underwent a maximum of 2 passages in proliferation culture prior to a novel SKP isolation procedure. Upon reaching confluency, SKPs intended for jejunal injection were transferred to T75 flasks containing NeuroCult® NS-A Basal Medium (Rat) with 10% Differentiation Supplement (StemCell Technologies) and 1× Antibiotic Antimycotic (ABAM) solution (Invitrogen), allowing 10–14 days to promote neuroglial differentiation. All cell cultures were incubated in 5% ambient CO_2 at 37 °C. Anti-GFP immunofluorescence and markers of enteric neurotransmitter synthesis were confirmed in a subset of fixed SKPs.

1.3. Aganglionosis model

Adult rats ($n = 9$) underwent midline laparotomy under inhaled isoflurane anesthesia, and jejunal aganglionosis was induced according to our previously described protocol [27]. The procedure resulted in a 1 cm-long isolated jejunal segment and a bypass jejunojejunostomy to restore intestinal continuity. Chemical denervation of the isolated jejunal segment was achieved by trans-serosal benzalkonium chloride (BAC, 0.2% w/v, Sigma-Aldrich) exposure for 20 min. The abdominal fascia was closed with 3-0 braided polyglactin, and the skin closed with 3-0 nylon suture. Postoperatively, a standard solid feeding regimen was reinstituted, and trimethoprim sulfa (TMS, 1% v/v) was administered via water bottle for 14 days. Among the 9 adult rats, 7 underwent SKP injection. The remaining 2 rats underwent immunohistochemical (IHC) evaluation for the presence of enteric ganglia and markers of neurotransmitter synthesis after 65 days.

1.4. SKP transplantation

GFP-expressing SKPs were liberated from differentiation culture and suspended in NeuroCult® NS-A Basal Medium (Rat) with 10% Differentiation Supplement (StemCell Technologies) (StemCell Technologies) with 15% v/v pH-neutralized rat tail collagen [17] in differentiation media and 2% v/v India ink (Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of 5×10^5 cells/mL. SKPs were injected 24–46 days after initial plating. Adult recipient rats ($n = 7$) underwent a second laparotomy under general inhaled anesthesia between 65 and 85 days after segmental jejunal denervation. The denervated segment was identified and SKPs were injected subserosally using a microinjector as described previously [27]. Injected segments were covered with omentum, and the abdomen and skin closed. After 1, 2, 7, 14, 21, and 28 days, the animals were euthanized and the experimental segments fixed for IHC evaluation.

1.5. SKP immunocytochemistry

A subset of SKPs in differentiation culture was fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) at 4 °C for 24 h and washed with phosphate-buffered saline (PBS). SKPs were then incubated overnight with anti-GFP IgY (1:400; Invitrogen) primary antibody. After serial PBS washings, the cells were subjected to goat anti-chicken IgY Alexa Fluor® 488 (1:200; Aves Labs, Tigard, OR) secondary antibody for 30 min. Immunofluorescence was visualized by microscopy (Leica Microsystems, Bannockburn, IL).

1.6. Jejunal immunohistochemistry

Jejunal specimens were fixed in 10% formalin (Fisher Scientific) for 24 h at 4 °C. Fixed tissue was embedded in paraffin wax and cut into 5-μm sections. Wax was removed in xylenes for 10 min, and the sections were washed serially in 100%, 95%, 70% ethanol (Fisher Scientific), and water for 2 min each. Slides were placed in citrate buffer (Biogenex, San Ramon, CA) for 20 min at 95 °C for antigen retrieval, then cooled for 30 min in running water. A PAP pen barrier was placed around each section (Vector Laboratories, Burlingame, CA). Tissue specimens were placed in a solution of 5% normal goat serum (Vector Laboratories) and 2% bovine serum albumin in PBS with 0.05% Tween-20 (PBS/T) for one hour at 25 °C to prevent nonspecific binding of secondary antibodies.

The specimens were incubated with primary antibodies diluted in PBS/T overnight at 4 °C in a humidified slide chamber. Primary antibodies to distinguish injected SKPs included anti-GFP IgY (1:400; Invitrogen) to identify SKP GFP expression, anti-neuron specific β-III-tubulin (TUJ1; 5 μg/ml; Abcam, Cambridge, MA) to identify mature neurons, and anti-glial fibrillary acid protein (GFAP; 1:400; Sigma-Aldrich, St. Louis, MO) to identify mature gliocytes. Primary antibodies to characterize engrafted neuronal phenotypes included anti-choline acetyltransferase (ChAT; 1 μg/μL; Abcam), anti-dopamine-β-hydroxylase (1:400; Abcam), anti-vasointestinal peptide (VIP; 1:400; Abcam), anti-neural nitric oxide synthase (nNOS) (1:50; Abcam), and anti-serotonin (1:50; Dako). Slides were washed three times in PBS/T for 30 min. Secondary antibodies were diluted in PBS/T and applied to tissue sections for 30 min. Secondary antibodies included goat anti-rabbit Alexa Fluor® 594, goat anti-mouse Alexa Fluor® 594 (all 1:200; Invitrogen), and goat anti-chicken IgY Alexa Fluor® 488 (1:200; Aves Labs, Tigard, OR). Slides were washed with PBS/T serially. Each section was treated with Prolong Gold with DAPI (Invitrogen) and covered with a glass coverslip. Histology slides were visualized under fluorescence microscopy (Leica Microsystems).

2. Results

2.1. SKP isolation

All three SKP cell lines were successfully cultured for characterization and transplantation. The average cell yield per isolation procedure was 8.2×10^6 cells. SKP doubling time in proliferation culture was 32.6 days on average. All SKPs in differentiation media demonstrated anti-GFP immunofluorescence, and expression of neurotransmitter synthesis markers was identified among differentiated SKPs *in vitro* (Fig. 1).

2.2. Aganglionosis model

All 9 adult Lewis rats underwent successful segmental jejunal isolation and chemical denervation. IHC stains of normal and denervated segments are shown in Fig. 2. Neurons and gliocytes were undetectable in denervated segments at all time points.