Transplanted skin-derived precursor stem cells generate enteric ganglion-like structures in vivo

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ABSTRACT

Introduction: Hirschsprung’s disease is characterized by a developmental arrest of neural crest cell migration, causing distal aganglionosis. Transplanted cells derived from the neural crest may regenerate enteric ganglia in this condition. We investigated the potential of skin-derived precursor cells (SKPs) to engraft and to differentiate into enteric ganglia in aganglionic rat intestine in vivo.

Methods: Adult Lewis rat jejunal segments were separated from intestinal continuity and treated with benzalkonium chloride to induce aganglionosis. Ganglia were identified via immunohistochemical stains for S100 and βIII tubulin (TUJ1). SKPs were procured from neonatal Lewis rats expressing enhanced green fluorescent protein (GFP) and cultured in neuroglial-selective media. SKP cell line expansion was quantified, and immunophenotypes were assessed by immunocytochemistry. Aganglionic segments underwent SKP transplantation 21–79 days after benzalkonium chloride treatment. The presence of GFP + cells, mature neurons, and mature glia was evaluated at posttransplant days 1, 6, and 9.

Results: Benzalkonium chloride-induced aganglionosis persisted for at least 85 days. Prior to differentiation, SKPs expressed S100, denoting neural crest lineage, and nestin, a marker of neuronal precursors. Differentiated SKPs in vitro expressed GFAP, a marker of glial differentiation, as well as TUJ1 and several enteric neurotransmitters. After transplantation, GFP + structures resembling ganglia were identified between longitudinal and circular smooth muscle layers.

Conclusion: SKPs are capable of engraftment, migration, and differentiation within aganglionic rodent intestine in vivo. Differentiated SKPs generate structures that resemble enteric ganglia. Our observations suggest that SKPs represent a potential gangliogenic therapeutic agent for Hirschsprung’s disease.

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1. Materials and methods

The following experimentation was conducted under UCLA protocol #2006-061 with the approval of the Institutional Animal Care and Use Committee.

1.1. SKP procurement and culture

Neonatal Lewis rat pups (3–5 days old) expressing enhanced green fluorescent protein (GFP) on the ubiquitin promoter were used for SKP proliferation and transplantation [20]. Native GFP fluorescence was confirmed by visualizing tail clippings from euthanized pups under ultraviolet light microscopy (excitation λ = 490 nm). SKPs from non-GFP neonatal Lewis rat pups were harvested to characterize in vitro neuronal differentiation potential.

SKPs were harvested according to the Biernaskie protocol [21]. A 1.5 × 1.5 cm² patch of dorsal skin was harvested and mechanically stripped of subdermal connective tissue and epidermis using a #10 scalpel blade. The dermis was diced into 1–3 mm² fragments and digested in 500 mg collagenase IV (Sigma-Aldrich, St. Louis, MO) dissolved in 10 mL Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA) for 1 hour at 37 °C. The dissociated cells were isolated and suspended in NeuroCult® NS-A Basal Medium (Rat) (StemCell Technologies, Vancouver, Canada). Cells were filtered through a 40-μm cell strainer (BD Falcon, San Jose, CA) and plated at a density of 5 × 10⁵ cells/mL. SKPs were transplanted at days 24, 41, or 94 after initial plating. Recipient rats (n = 9) underwent a second laparotomy under isoflurane anesthesia at post-BAC treatment days 21–79. The isolated BAC-treated segments were identified and meticulous adhesiolysis was performed. The SKP suspension was injected suberosally in a fanning linear fashion using a microinjector with a 0.5-inch long 33-gauge needle (Hamilton, Reno, NV). Approximately 100–200 μl of suspended SKPs were injected into each segment. After injection, the isolated segment was wrapped with mobilized omentum, replaced into the peritoneal cavity, and the abdomen and skin were closed. At 1, 6, or 9 days posttransplant, the animals were euthanized and the

1.2. Aganglionosis model

Adult female Lewis rats (n = 18) were purchased from Charles River Laboratories (Wilmington, MA) for the purpose of enteric nervous ablation and SKP transplantation. Isoflurane anesthesia was administered, midline laparotomy performed, and the small bowel eviscerated. A 1-cm segment of jejunum was isolated from continuity, preserving its vascular supply, approximately 10–15 cm distal to the ligament of Treitz. The jejunal segment was wrapped with sterile gauze saturated with benzalkonium chloride (BAC, 0.2% w/v, Sigma-Aldrich) for 20 minutes. A jejunojejunostomy was performed with 6–0 polypropylene suture to restore intestinal continuity, excluding the isolated segment. The treated segment was then irrigated with sterile 0.9% normal saline and the ends sutured closed with 6–0 polypropylene. The omentum was wrapped around the isolated segment, and the viscera were replaced into the peritoneal cavity. The abdomen was closed using 3–0 polyglactin braided suture, and the skin closed with 3–0 nylon suture. Rats were administered trimethoprim sulfa (TMS, 1% v/v) for 14 days, postoperatively. For comparison, two additional adult female Lewis rats underwent jejunal segment isolation without BAC treatment.

pH-neutralized rat tail collagen was prepared using our laboratory’s previously-described protocol [11]. SKPs were liberated and suspended in differentiation media with 15% v/v neutralized rat tail collagen in differentiation media and 2% v/v India ink (Becton, Dickinson and Company, Franklin Lakes, NJ) at a density of 5 × 10⁵ cells/mL. SKPs were transplanted at days 24, 41, or 94 after initial plating. Recipient rats (n = 9) underwent a second laparotomy under isoflurane anesthesia at post-BAC treatment days 21–79. The isolated BAC-treated segments were identified and meticulous adhesiolysis was performed. The SKP suspension was injected suberosally in a fanning linear fashion using a microinjector with a 0.5-inch long 33-gauge needle (Hamilton, Reno, NV). Approximately 100–200 μl of suspended SKPs were injected into each segment. After injection, the isolated segment was wrapped with mobilized omentum, replaced into the peritoneal cavity, and the abdomen and skin were closed. At 1, 6, or 9 days posttransplant, the animals were euthanized and the

![Fig. 1. SKPs in proliferation media. A) Native GFP fluorescence in live SKPs; B) GFP + SKPs demonstrating attenuated native fluorescence upon fixation; C) anti-GFP immunofluorescence in fixed SKPs; D) S100 expression (green); E) nestin expression (red); F) merged S100 and nestin expression. Nuclei in panels D–F appear blue from DAPI, and scale bars represent 100 μm.](image-url)
injected segments collected and fixed for immunohistochemistry (IHC). Recipient muscularis thickness and the presence of native ganglia were compared among segments injected at earlier versus later time points after BAC treatment.

1.4. SKP immunocytochemistry

A subset of SKPs in culture was fixed overnight in 10% formalin (Fisher Scientific, Pittsburgh, PA) at 4 °C. Cells were washed with phosphate-buffered saline (PBS). SKPs were incubated overnight with the following primary antibodies: anti-S100 (1:200; Dako, Carpenteria, CA), anti-nestin (1:200; Abcam, Cambridge, MA), anti-neuron specific β-III-tubulin (1:200 Abcam), anti-glial fibrillary acid protein (1:400; Sigma-Aldrich), and anti-GFP IgY (1:400; Invitrogen).

A subset of GFP-negative SKPs was cultured in differentiation media for 7 days prior to formalin fixation as above. These SKPs were incubated overnight with the following primary antibodies: anti-vasointestinal peptide (1:400; Abcam), anti-neuronal nitric oxide synthase (nNOS) (1:50; Abcam), and anti-dopamine-β-hydroxylase (1:400; Abcam), anti-serotonin (1:50; Dako), and anti-choline acetyltransferase (ChAT; 1 μg/μl; Abcam).

Cells were washed three times with PBS, and fluorophore-tagged secondary antibodies were administered to the cells for 30 minutes. Secondary antibodies included goat anti-rabbit Alexa Fluor® 488, goat anti-mouse Alexa Fluor® 488, goat anti-rabbit Alexa Fluor® 546, donkey anti-mouse Alexa Fluor® 555, goat anti-rabbit Alexa Fluor® 594, goat anti-mouse Alexa Fluor® 594 (all 1:250; Invitrogen), and goat anti-chicken IgY Alexa Fluor® 488 (1:200; Aves Labs, Tigard, OR). Cell plates were visualized via fluorescence microscopy (Leica Microsystems, Bannockburn, IL, and Zeiss, Thornwood, NY).

1.5. Jejunal immunohistochemistry

After tissue procurement, retrieved jejunal segments were fixed in 10% formalin (Fisher Scientific) for 24 hours at 4 °C. Fixed tissue was embedded in paraffin wax and cut into 5-μm sections. Paraffin was dissolved in xylenes for 10 minutes and rehydrated in serial washes for 2 minutes each in 100%, 95%, 70% and 0% ethanol (Fisher Scientific). Antigen retrieval was performed in citrate buffer (Biogenex, San Ramon, CA) for 20 minutes at 95 °C. Slides were cooled in a running water bath for 30 minutes. A hydrophobic barrier was applied to each section with a PAP pen (Vector Laboratories, Burlingame, CA). Nonspecific secondary antibody binding was blocked by incubating the slides 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 room temperature. Primary antibodies were anti-GFP IgY (1:400; Invitrogen), anti-S100 (1:200; Dako), anti-neuron specific β-III-tubulin (5 μg/ml; Abcam), and anti-glial fibrillary acid protein (1:400; Sigma-Aldrich). These primary antibodies were selected to maximize the sensitivity of glial and

Fig. 2. SKPs in differentiation media. A) GFAP expression; B) TUJ1 expression; C) ChAT expression; D) dopamine-β-hydroxylase expression; E) VIP expression; F) nNOS expression. Arrows indicate clusters of fluorescent granules, and scale bars represent 100 μm.
neuronal cell detection. Antibodies diluted in PBS/T were incubated with slides overnight at 4 °C in a humidified slide chamber. Slides were washed for 30 minutes in PBS/T three times. Secondary antibodies were diluted in PBS/T and exposed to the tissue sections for 30 minutes at room temperature. Slides were washed again for 30 minutes with PBS/T three times. Secondary antibodies included goat anti-rabbit Alexa Fluor® 488, goat anti-mouse Alexa Fluor® 488, goat anti-rabbit Alexa Fluor® 546, donkey anti-mouse Alexa Fluor® 555, 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). Each section was treated with Prolong Gold with DAPI (Invitrogen) and covered with a glass coverslip. Histological slides were visualized under fluorescence microscopy (Leica Microsystems and Zeiss).

2. Results

2.1. SKP cell culture

Five out of six SKP cell lines were successfully cultured for characterization and transplantation. One SKP cell line yielded insufficient cells to sustain proliferation. The average SKP doubling time in culture was 16.6 days. In proliferation media, GFP expression was ubiquitous in SKPs; however, native GFP expression was considerably attenuated upon fixation (Fig. 1A–C). Increased time in culture was associated with less intense anti-GFP immunofluorescence (data not shown). Most (>85%) SKPs in proliferation media expressed S100, a marker of neural crest lineage, and approximately 15% expressed nestin, a marker of neural precursor cells (Fig. 1D–F).

After 7–14 days in differentiation media, approximately 20% of SKP cells expressed GFAP and 10% expressed neuron specific β-III-tubulin (TUJ1) (Fig. 2A and B), indicating glial and neuronal differentiation, respectively. SKP-derived neurons expressed ChAT, dopamine-β-hydroxylase, VIP, and nNOS, markers of enteric neurotransmitter synthesis (Fig. 2C–F). SKPs in differentiation media expressing serotonin (5-HT) were not visualized.

2.2. Aganglionosis model

Sixteen out of eighteen adult Lewis rats were successfully treated with BAC. Two rats succumbed to respiratory insufficiency at the time of BAC treatment. Histologic sections of normal, isolated, and BAC-treated isolated jejunal segments are shown in Fig. 3. Segmental isolation alone (without BAC treatment) resulted in muscularis propria hypertrophy with easily detectable myenteric and submucosal ganglia (Fig. 3C and D).
BAC treatment obliterated enteric ganglia at all time points observed. Muscular hypertrophy and aganglionosis persisted for at least 85 days after BAC treatment (Fig. 3E and F).

2.3. SKP transplantation

Nine adult Lewis rats received SKP cell injections at 21–79 days post-BAC treatment. Smooth muscle hypertrophy was insufficient to accommodate intestinal injection prior to 21 days after BAC treatment. There was greater hypertrophy at later time points (data not shown). India ink-stained collagen aggregates indicated injection sites on hematoxylin and eosin (H&E) staining (Fig. 4A). India ink was present within the submucosa, smooth muscle, serosa, and extraperitoneal fat layers of several sections. Well-circumscribed, elongated structures comprised of GFP + cells coexpressing GFAP, S100, and TUJ1 were identified in the intermuscular layer of 7 rats after

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**Fig. 4.** Posttransplantation jejunum. A) H&E staining of BAC-treated jejunum, 100× magnification. Arrowheads indicate myenteric neuroglial structure, arrows indicate India ink in extraperitoneal tissue. B) H&E staining of BAC-treated jejunum, 200× magnification. Arrowheads indicate myenteric neuroglial structure. C) GFP (green) and S100 (red) expression, 200× magnification. D) GFP (green) and S100 (red) expression, 200× magnification. E) GFP (green) and GFAP (red) expression, 200× magnification. F) GFP and GFAP expression, 630× magnification. G) GFP (green) and TUJ1 (red) expression, 200× magnification. H) GFP and TUJ1 expression, 630× magnification. Scale bars represent 100 μm. CM: circular smooth muscle, LM: longitudinal smooth muscle, E: mucosal epithelium.
transplantation (Fig. 4C–H). These structures were evident at all posttransplant intervals, and with all SKP cell lines transplanted. These structures were not evident in other bowel layers. Differences in SKP age were not associated with observable changes in posttransplant tissue morphology or immunophenotype.

3. Discussion

SKPs are capable of migration, differentiation, and engraftment within living aganglionic recipient intestine. We observed the presence of ganglia composed of GFP-expressing mature neurons and glial cells in recipient aganglionic jejunum following SKP transplantation. Our findings suggest that SKPs comprise a potential source of autologous cell therapy to reconstitute the enteric nervous system. When cultured in differentiation media, our SKPs recapitulated the observations of Kwok and colleagues, who demonstrated S100, GFAP, Tuj1, VIP, NOS, and ChAT expression among human SKPs cultured in differentiation media for 14 days [19]. These results are promising, in that SKPs are capable of differentiating into glial and neuronal subtypes common to enteric ganglia. These in vitro results also signify that our SKPs represent a mixture of cell types. The clinical yield of a mixed-cell culture may be superior to that of a clonal culture. Liebmann and colleagues observed that SKP-derived neurons proliferate more efficiently when cocultured with glial cells [18]. While the growth characteristics and immunophenotypes of each cell line in our study were somewhat variable, we observed little dissimilarity in posttransplant outcomes. The intercellular activity within a mixed culture may be instrumental in translating cell harvest to cell therapy. Nevertheless, SKP gene expression profiles and clonal characterization over time and in growth factor–enriched media should be the subject of further experimentation.

We find the migration and differentiation behavior of transplanted SKPs particularly interesting. We observed that, whether injected into the extracellular, subepithelial, intramuscular, or submucosal layers, SKP-derived neurons and glia localize to the precise layer normally occupied by the myenteric plexus within days. In one study, neural crest-derived cells transplanted into the peritoneal cavity were detected within the intestinal wall [22]. Within this context, our observations support the notion that transplanted precursor cells are impelled to migrate by the recipient tissue milieu. Other studies have also demonstrated the capacity of SKPs to transdifferentiate into peripheral nervous tissue, corneal epithelium, and skin, depending in large part upon recipient factors [23–25]. Specific factors within the intestinal wall microenvironment that contribute to SKP migration and differentiation remain indistinct. In Hirschsprung’s disease, the unidentified elements that precipitate neural crest migratory arrest may also impede the migration of transplanted cells; however, allogeneic myoneural neuronal growth has been demonstrated in Hirschsprung’s colon [26].

The function of recipient intestinal tissue after SKP cell transplantation necessitates further investigation. In addition to differentiation, migration, and engraftment, transplanted SKPs must undergo physiologic orientation, synaptogenesis, and target innervation to generate coordinated intestinal motility and glandular stimulation. Specific subjects of future studies should include diagnostic indicators of human Hirschsprung’s disease such as pretransplant and posttransplant expression of calretinin and acetylcholinesterase, SKP-derived neuron neurotransmitter production, in vivo action potential conduction, enteric plexus architecture, amplitude and direction of peristaltic muscle contraction, and alimentary function of recipient tissue.

Acknowledgments

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[14] Fernandes KJL, Kobayashi NR, Gallagher CJ, et al. Analysis of the neurogenic factors within the Hirschsprung’s disease. The function of recipient intestinal tissue after SKP cell transplantation (Fig. 4C–H). These structures were evident at all posttransplant intervals, and with all SKP cell lines transplanted. These structures were not evident in other bowel layers. Differences in SKP age were not associated with observable changes in posttransplant tissue morphology or immunophenotype.

**Discussion**

Discussant: Robert Cowles, MD:

‘I really great work, and the idea to actually give back an enteric nervous system is very appealing. I just wanted to ask you a couple of questions about your immunohistochemistry and the first is how do you think that your injection gets the cells into the right location for potential future function meaning how does it really get into that myenteric plexus between the outer and the inner muscular layers. The second thing is did you show (and I wasn’t really clear based on your photos) whether a submucosal plexus
also forms or whether some of your cells actually make into the submucosal area because there is an outer myenteric plexus and there is an inner submucosal plexus?

Response: Dr. Wagner: Thank you very much for your questions. I'll attempt to answer the second one first. We didn't really see too many of our same beautiful myenteric structure types in the submucosal plexus. There are some cells that migrate to that layer, but they don't form very large ganglia like the first one. As for your first question, how does cell migration occur, I can only speculate that there may be some remnant structures of axons that exist that may be giving some sort of mechanical cue to the cells that are injected. What is interesting is that it doesn't really matter where the cells are injected even outside of the bowel wall, we have seen cells that are injected in the fibrotic tissue that still seem to migrate and if you look at the cells one day after transplantation you can see a smattering of them through the muscular layer, and they somehow make their way into the myenteric plexus.

Discussant: Dr. Agostino Pierro, Toronto, Canada: I would like to congratulate you for this fantastic study obviously with very important clinical potential. I would like to ask you why you chose the jejunum because it is known that the distribution of the ganglion cells is different from the large bowel, and that is where the disease is primarily the problem. The second question is how far from your injection site does the distribution of these cells occur? Very beautiful work.

Response: Dr. Wagner: Thank you for your questions. We chose the jejunum because it's just simply a more technically easy model system to make. The wall of the colon in rats is extremely thin, and we depend on the hypertrophy effect of benzalkonium to generate enough of a landing point for the cytoinjection. Also we can easily perform a bypass next to this isolated segment. The rat does fine after several days and even weeks after the operation.

Then the second question, how far from the site of injection do we see the cells, well we have only used a 1-cm long piece of jejunum for each of the injections, and so really have injected across the entire length of it to try to generate as much as we possibly can. I really can't comment on exactly how far they migrate, but also the number of ganglia is significantly less than what you would see in normal jejunum.

Discussant: Dr. Gail Besner, Columbus, OH: I want to commend you on that really elegant study, beautifully presented as well. You used chemical ablation of the enteric nervous system here and I was wondering if you have been able to recapitulate these results using for example genetically engineered mice like EDNRB knockout mice which is another accepted model of Hirschsprung's disease.

Response: Dr. Wagner: Excellent point and excellent question, and yes that is going to probably be a target of a future study. One of my colleagues uses an endoscopic model of cell injection using EDNR negative mice and he can probably try the same thing except unfortunately this particular model system doesn't work so well in mice. They don't tolerate a laparotomy, but that is a target of our future study. Thank you very much.