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Trans-differentiation of a Duodenal Phenotype on Duodenal Transplantation of Different Normal Tissues in F344 Rats

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ABSTRACT

The mechanisms regulating stem cell differentiation and self-renewal are largely unknown. Our ultimate goal is to be able to regulate somatic stem cell differentiation and proliferation. In the present study the ability of trans-differentiation was studied when different normal tissue types were transplanted into the duodenum in rats. Pieces of ear (skin), bladder, trachea, diaphragm, pyloric gland, and forestomach from 8-week old GFP-F344 rats were transplanted into the duodenum of F344 rats. Goblet cells with alcian-blue PAS positive mucin and brash border with alkaline phosphatase (ALP) activity appeared in tissues implanted into the duodenum. In addition, GFP-positive duodenal mucosa was observed in all cases by immunohistochemical staining. Moreover, the GFP-positive cells were found to carry the GFP transgene by PCR analysis, indicating that the bladder, trachea, ear (skin), diaphragm, pyloric gland, and forestomach tissues showed a multipotential ability for differentiation. These results indicated that stem cells within tissues have a multipotential ability, trans-differentiating into different organs when transplanted into different environments.

Key words: *Transplantation, Hetero-tissues, Duodenum, F344 GFP Rats*

The mechanism underling a key property of stem cells, such as maintenance of stemness and cellular differentiation, is still not understood. Mesenchymal cells are a well-differentiated population of bone marrow-derived non-hemopoietic cells with multipotent properties. The medical interest in such multipotency rests in the potential of such cells to repair damaged tissues. Several recent reports suggest that there is far more plasticity than previously believed in the developmental potential of many different adult cell types. Adult stem cells have been reported from several tissue sources, including the central nervous system[32], bone marrow[12,21,22], retina[16], brain[6], hair follicle[24], inner ear[13], adipotic tissues[8,14], oral mucosal epithelium[19], liver[25], skeletal muscle[2] and skin[9,11].

We have reported that intestinal metaplasia can be induced by X-irradiation in rats[26], and have proposed that an elevation of the pH of gastric juice due to the disappearance of parietal cells in the fundic gland mucosa is one of the principal factors responsible for its development[7,26,28,31]. We also found that grafted colon mucosa differentiates into pseudogastric mucosa in the gastric region and that stomach grafts differentiate into intestine with goblet cells in the duodenum[27]. We also found that when the bladder was transplanted into the duodenum, goblet cells with alcian-blue positive mucin appeared and alkaline phosphatase positivity was noted in the bladder[29]. Our ultimate goal is to be able to regulate somatic stem cell differentiation and proliferation.

Currently, we have demonstrated that organ-specific stem cells in normal non-liver tissues differentiate into hepatocytes when they are transplanted into injured liver[30]. In the present study, to further elucidate the multipotential ability of tissue-specific stem cells when they are transplanted into different environments, different tissues from GFP-F344 rats were transplanted into the duodenum in recipient rats. Our results showed that normal tissues from ear, bladder, trachea, diaphragm, pyloric gland, and forestomach

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revealed a multipotential ability for differentiation into duodenum.

MATERIALS AND METHODS

Animals

Donors: Irrespective of the approach used to obtain stem cells, the fates of adult stem cells following tissue transplantation can be traced by labeling donor cells with green fluorescence protein GFP, a non-secreting cytosolic protein, which appears green under UV light. The plasmid construct carrying GFP is driven by CAG promoter. The fate of any cell expressing GFP is easy to investigate by UV light or by immunohistochemistry in combination with anti-GFP antibody. The *Eco*RI fragment of full-length EGFP cDNA was introduced into a pCAGGS expression vector containing chicken beta-actin promoter and cytomegalovirus enhancer, beta-actin intron and bovine globulin poly-adenylation signal[20]. Transgenic rat lines were produced by injecting the purified linealized pCAG-EGFP plasmid DNA into F344/Du Crj (Fisher) rat fertilized eggs. In total, 320 DNA-injected eggs were transplanted to pseudo-pregnant rats, resulting in 46 newborns. The incorporation of the transgene was examined by placing 1-day-old pups under a fluorescent microscope. Five were found to be transgenic.

Recipients: Eight-week-old F344 rats of both sexes were purchased from Charles River, Japan Co. Ltd. (Hino) and housed in polycarbonate cages, 4 animals per cage, under constant conditions of temperature (24 ± 2℃) and humidity (50 ± 10%), with a 12 hr light/12 hr dark cycle. They were fed commercial diet MF (Oriental Yeast, Co. Ltd., Tokyo) and were provided with tap water *ad libitum*. The animals were maintained under the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals", established by Hiroshima University.

Operation

Donor rats were sacrificed by ether. Ear section with less hair, bladder sliced to form a sheet, 7 mm of trachea, diaphragm, pyloric gland and forestomach cut horizontally to make squares, were cut from resected organs of male rats and also female donors into male recipients under pentobarbital and ether anesthesia. The tissue specimens were then grafted into the whole layer of the duodenum 2 cm from the pyloric ring of age-matched isogeneic rats by a 7-0 needle with strings (Nescosuture, Aswell, Osaka, Japan).

Pathology

Animals were autopsied after sacrifice 1, 3 or 6 months after the surgery. The duodenum was removed, opened along the long axis and extended on cardboard for inspection. The tissues were fixed in 10% neutral formalin for routine processing, paraffin embedding and alcian-blue periodic acid-Schiff (PAS), hematoxylin-eosin and alkaline phosphatase (ALP, Dako Fuchin substrate-chromogen system, Code K0624, Dako North American Inc, Ca) staining of tissue sections.

Immunohistochemistry

Three-μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies, anti-GFP antibody (diluted 1:200; Abcom ab6556, England), anti-PCNA (diluted 1:50; FL-261 Santa Cruz Biotechnology, CA) or mouse anti-CDX-2-88 (diluted 1:50; BioGenex, CA) for 1 hr at room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca, Catalog No. PK-8800) and peroxidase conjugated streptoavidin complexes. Peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 15 min. At the final step, the sections were counterstained with hematoxylin for 1 min. GFP-positive site was cut by the microdissection apparatus and GFP gene was detected.

Detection of transgene expression

GFP-positive cells in the duodenum engrafted with tissue pieces of trachea, bladder and ear were harvested using a LM200 PixCell LCM System (Arcturus Bioscience, Inc., Mountain View, CA). Tissue was collected on TF-100 caps (Arcturus) containing transfer film from two or three sections for each sample. DNA was extracted using SepaGene solution (Sanko Jyunyaku Co. Ltd.) according to the manufacturer's protocol. PCR detection of EGFP cDNA was performed with the TaKaRa Ex TaqTM (TaKaRa SHUZO Co., Ltd.) following standard procedures, using the primers recommended by Clontech: forward, 5(CGT CGC CGT CCA GCT CGA CCA G 3; reverse, 5 CAT GGT CCT GCT GGA GTT CGT G 3. As a control, quality of RNA was assessed by β-actin primers: forward, 5 AGA GCA AGA GAG AGG TAT CCT G 3; reverse, 5 AGA GCA TAG CCC TCG TAG AT 3.

RESULTS

Implantation of somatic tissues into duodenum

The tissue specimens of male and female F344 rats carrying the *GFP* gene were grafted into the whole layer of the duodenum 2 cm from the pyloric ring of age-matched isogenic male and female rats, respectively, (male donor transplanted to female recipient and female to male). Both transplantations showed the same histological findings so the results were combined. All donor tissues includ-

Table 1. Number of recipient rats used histological examinations.

Organ	1 month after transplantation	3 months after transplantation	6 months after transplantation
Trachea	3	9	3
Bladder	3	10	1
Ear	4	4	3
Diaphragm		12	
Glandular stomach			6
Forestomach			5

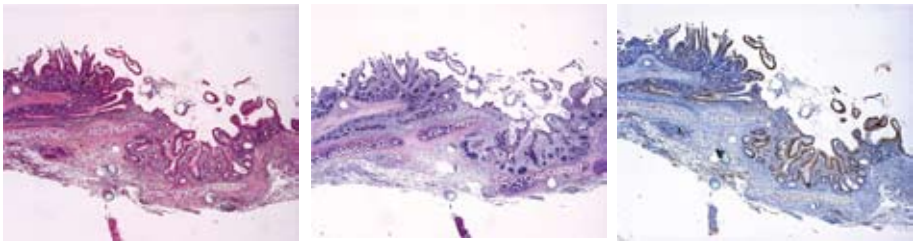

Fig.1-1 **Fig.1-2** **Fig.1-3**

Fig.1. One month after ear transplantation, ×40
1-1. Intestinal-like structure, HE staining
1-2. Many goblet cells with alcian blue-PAS positive mucin and brash border, alcian blue-PAS staining
1-3. GFP positive intestinal-like structures, GFP immunostaining,

ing ear, trachea, bladder, diaphragm, glandular stomach, and forestomach were successfully engrafted into both sexes (Table 1). Seventy-three animals were operated on and ten were dead 1 day after operation. One month after trachea transplantation into the duodenum, an implanted piece with strings still remained. All donor pieces were found upon autopsy in recipient duodenum.

Histologically, cell infiltrations, ulcer, fibrosis or granulation were found in transplanted site strings. Intestinal-like epithelia (Fig. 1-1) which had goblet cells containing alcian-blue-PAS positive mucin (Fig. 1-2), GFP positive (Fig. 1-3) and brush-border with ALP activity had appeared. PCNA positive cells or mitosis were found in the lower part of the crypt which existed in the same position in the intrinsic crypt. Near the transplanted site, ulcer or fibrosis, low-growing intestinal structures or only crypts without villus and Paneth cells appeared.

Ear

Ear cartilages remained 6 months after transplantation and their edges changed by mineralization from 3 months. Squamous cell mucosa and subaceous epithelia were observed until 3 months. Hairs were observed only 1 month after implantation. Cell infiltration and ulcer appeared around the squamous epithelia.

One month after transplantation, intestinal-like structures had crypts and villus with goblet cells and ALP, GFP-positive, and PCNA positive cells appeared. Only crypts appeared near ear cartilage. Squamous cell component was trans-differentiated into one layer intestinal-like mucosa continuous with alcian-blue PAS positive goblet cells and ALP positive brush border. Three months after transplantation, intestinal-like mucosa with GFP-positive cells was elongated from the squamous cell basement membrane into the intestine component.

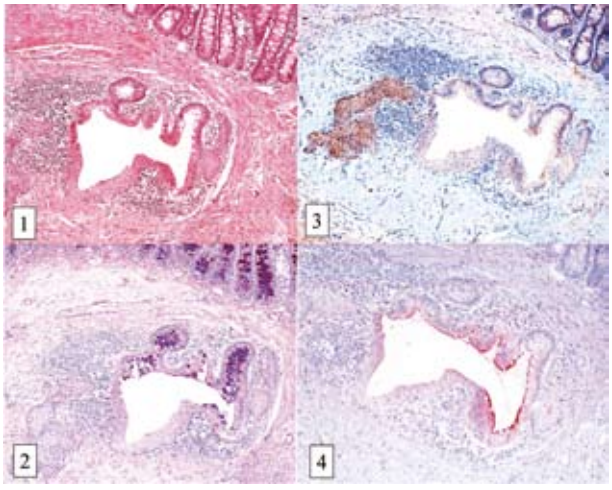

Fig.2. Three months after bladder transplantation, × 100
2-1. Squamous epithelium is re-differentiated into intestinal structure, HE staining
2-2. Many goblet cells appear, alcian blue-PAS staining
2-3. GFP staining
2-4. ALP staining

Bladder

Transitional epithelia were trans-differentiated into squamous epithelium, which appeared as ALP positive cell membrane without goblet cells. PCNA positive cells were also found from 1 month after transplantation. Three of nine implanted specimens were observed with severe proliferation of squamous epithelia after 3 months. Squamous epithelia with goblet cells, GFP-positive and brush border (Figs. 2-1, 2-2, 2-3, 2-4) appeared in quantity from 3 months after transplantation. Some of the remaining transplanted organs appeared with a cyst of one thin layer of cells. Six months after operation squamous epithelia decreased and one cell layer cysts were found.

Trachea

The trachea cartilages and epithelium still remained until 6 months after transplantation. Cell infiltration around cartilages was severed after one month but decreased after 3 months and small amounts remained at 6 months. The trachea epithelium was trans-differentiated into intestinal-like structures with alcian-blue PAS positive goblet cells and ALP positive brush border near cartilages or appeared as ALP positive cytoplasm. Three months after transplantation, squamous cell membranes had ALP positive. Six months after transplantation, negative staining of cdx-2, which is one of the intestinal markers, was shown in squamous cells without goblet cells.

Diaphragm

Three months after transplantation, only degenerated muscle-like structure in the musculis mucosa was detected. GFP positive intestinal-like crypts were observed widely near strings.

Pyloric gland

Six months after transplantation, mucous cells were not found and two of six appeared calcified in mucosa. GFP and cdx-2 positive intestinal-like crypts were observed widely near strings.

Forestomach

Three of six specimens still remained in the squamous epithelium. They were trans-differentiated into intestinal type structures with goblet cells and GFP positive and only crypts with many goblet cells near the squamous epithelia and strings.

Appearance of GFP-positive cells and their characterization

One, three and six months after transplantation, the implant sites were harvested and their tissue sections were analyzed. To monitor the presence of donor derived-cells, tissue sections were analyzed for the GFP-positive cells by immunohistochemical staining. To further elucidate whether these GFP-positive cells originated from donor tissues of F344/DuCrj EGFP rats, PCR analysis coupled with LCM was performed. PCR analysis revealed that GFP-positive cells were found to carry the GFP transgene (Fig. 3). It was also confirmed that the GFP-positive locus and cartilage had GFP transgene.

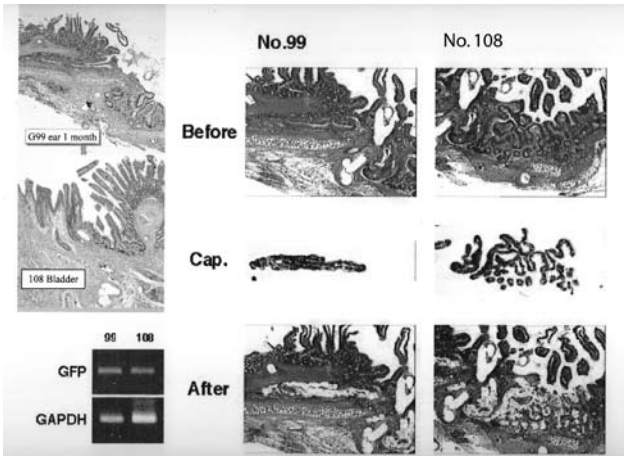

Fig.3. Microdissection of ear (No. 99) and bladder (No. 108) 1 month after transplantation.
PCR analysis coupled with LCM system was performed. PCR analysis revealed that GFP-positive cells were found to carry the GFP transgene.
Before; original tissue; Cap; harvested tissue; After; tissue after microdissection using LCM system

DISCUSSION

In the present experiment, the ear, trachea, bladder, diaphragm, pyloric gland and forestomach grafts transplanted into the duodenum were able to be trans-differentiated into the duodenum. It seems that pyloric mucosa and diaphragm (muscle) were more easily trans-differentiated than squamous cells and trachea epithelia. Further studies of sequential observation are required to probe this phenomenon. Previously we found that when colon tissues were transplanted into the fundus, periodic acid Schiff positive cells appeared near edges of the grafts[1,17,18], and also that pepsinogen positive crypts with alcian-blue positive goblet cells were generated in gastric mucosa implanted into the duodenum[27]. On the other hand, the esophagus epithelium can undergo metaplastic change to glandular epithelium[29] or duodena[15], glandular epithelium can become intestinal epithelium[10,15], small intestinal epithelium can change to glandular epithelium[3], and large intestinal epithelium to small intestinal epithelium[4,23,33], under the influence of different gastrointestinal tract diseases. Thus tissue differentiation in the gastrointestinal tract appears to be malleable. Moreover, we reported that when bladder

grafts were transplanted into the duodenum, they trans-differentiated to goblet cells[29]. However, we were notable to distinguish between grafts and recipient organ in previous experiments. In the present experiment structures between the grafts and transplanted sites were distinguished by immunohistochemical staining, and confirmed by the GFP gene. It was discernible that the graft differentiated into the recipient tissue. Recently we observed that when other organs such as heart, kidney, skin and brain are transplanted into the liver, all could be trans-differentiated into liver[30]. Heart transplanted into kidney could trans-differentiate into the kidney, and ear, bladder or colon transplanted into fundic gland trans-differentiated to gastric mucosa. Moreover, small intestine transplanted into bladder could trans-differentiate into bladder (Watanabe et al, unpublished data). Transplant experimentation like that reported here can be of assistance in clarifying the role of the tissue-environment. Kim et al demonstrated similar results that transplanted autologous skin epithelial stem cells can be trans-differentiated into tracheal epithelial cells and chondrocytes[11]. Bellayr et al reported that skeletal muscle-derived stem cells differentiated into hepatocyte-like cells and could aid in liver regeneration[2]. Kuroda et al found that the cells (Muse cells) which are stress-tolerant and can be isolated from cultured skin fibroblasts or bone marrow stromal cells or directly from bone marrow aspirates can self-renew, differentiate into endodermal, ectodermal and mesodermal cells, and are normally maintained in adult human tissues[12].

Thus, our results indicate that adult stem cells may have multipotential ability for differentiation and are able to trans-differentiate when transplanted into different environments.

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