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ORIGINAL ARTICLE

Cellular Dynamics after Injection of Mesoderm-Derived Human Embryonic Kidney 293 Cells and Fibroblasts into Developing Chick Embryos

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This study was conducted to compare localization of transformed or differentiated cells after injection into developing chick embryos. Mesoderm-derived chicken embryonic fibroblasts (CEFs), retrieved from normal tissues and artificially transformed human embryonic kidney (HEK) 293 cells, were injected into the dorsal aorta of stage 17 embryos, incubated for 60 h, and post-injection survival and tissue localization after injection were monitored. Overall survival rates were 43% to 57%, and there was no significant difference between the two cell types (P=0.4453). Migration into various tissues was observed after injection of the HEK 293 cells, and this was greatly reduced after CEF transfer ($P < 0.0127$). Tumorigenic activity was detected in the HEK 293 transferred cells and the major organ colonized was the highly vascularized yolk sac. From these results, we suggest that cell transformation alters post-injected migration activity of cells at organogenesis. **(J Cancer Prev 2014;19:68-73)**

Key Words: Animal cancer model, Chicken embryo, Tumorigenesis, Differentiation

INTRODUCTION

As they are easily accessible and feasible, being derived from an *in vitro*-like *in vivo* system, avian species have become one of the major model animal systems.[1] The developing chick embryo is one of the most powerful models for investigation of cell plasticity and their properties in organogenesis, and various models of xenotransplantation, development, immunity and even oncogenesis have been suggested.[2-9] These models continue to contribute to the expanding industrial applications of chick embryos in bioreactors, as well as to clarifying developmental events.[10-15]

In this study, we monitored the migration activity of cells derived from normal tissues and artificially transformed cells. Recent advances in cell biotechnology have enabled the use of genetically or cytologically manipulated cells, which subsequently undergo transformation. Cell transformation has been reported to influence immortalization, proliferation and self-renewal, as well as oncogenesis. In a previous study using mouse fibroblasts, transformed cells retained both their stem cell nature and tumorigenic activity (Gong et al., 2013).[16] This study was conducted to compare the migration activity of normally differentiated cells (chicken embryonic fibroblasts; CEF) and artificially transformed cells (human embryonic kid-

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ney, HEK 293 cells). This comparison will facilitate understanding of how cell transformation influences cellular activity after injection into developing chicken embryos. Stage 17 embryos were used for cell transplantation, and post-injection migration and localization after cell injection were monitored.

MATERIALS AND METHODS

1. Experimental animal care and management

White Leghorn (WL) chickens were used as the experimental model. The care and use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-070823-5), Korea. The procedures for chicken management, reproduction, and sacrifice adhered to the standard operating protocols of Laboratory of Animal Genetic Engineering, Seoul National University.

2. Transfection and selection of the GFP-expressing HEK 293 cell line

HEK 293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1× antibiotic-antimycotic solution (Invitrogen). HEK 293 cells were cultured in an incubator at 37°C with an atmosphere of 5% CO_2 and 60-70% relative humidity. HEK 293 cells were subcultured onto 0.1% gelatin-coated culture plates at 5-to-6-day intervals by 0.05% trypsin-EDTA treatment (Invitrogen).

To establish GFP-expressing HEK 293 cells, expression vectors containing the GFP gene, together with the immediate-early cytomegalovirus (CMV) enhancer/promoter and the neomycin-resistance (Neo^R) gene, controlled by the Simian virus 40 (SV40) promoter, were transfected into the HEK 293 cells and selected with 300 μg/ml G418. GFP expression in the HEK 293 cells was detected under a fluorescence microscope. The basic CAGG-PBase (pCyL43) and *piggyBac* transposon (pCyL50) vector frames were donated by the Sanger Institute (http://www.sanger.ac.uk).

3. Injection and screening of GFP-expressing HEK 293 cells in chicken embryos

GFP-expressing HEK 293 cells (1×10^5) were injected into the dorsal aorta of stage 17 recipient embryos, and incubated for 60 h. A small window was opened at the pointed end of recipient eggs, and a 2 μl aliquot containing the donor cells was injected into the dorsal aorta using a micropipette. Each window was sealed with paraffin film after injection, and the injected eggs were incubated pointed-end down, until screened for development and cell migration. As a control, GFP-expressing chicken embryonic fibroblasts (CEFs) were injected into the same number of eggs.

4. Cytological examination and histochemical staining

Cytological examination to detect GFP-expressing neoplasms in the recipient chicks was carried out using a specialized excitation lamp and goggles with a detection filter (BLS Ltd., Budapest, Hungary). A 460-495 nm wavelength was used for excitation and GFP signals were detected with a 500-550 nm emission filter. Samples of the tumor-like tissue were fixed in 4% paraformaldehyde for 24 h. The fixed tumors were treated with 70% ethanol for 24 h and then dehydrated and embedded in Paraplast Plus (Leica Microsystems, Wetzlar, Germany). Paraffin-em-

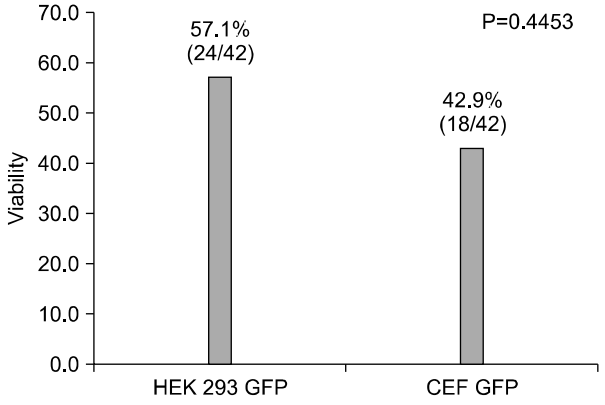

Fig. 1. Survival of chicken embryos after injection of green fluorescent protein (GFP)-transfected human embryonic kidney (HEK) 293 cells or GFP chicken embryonic fibroblasts (CEFs). Cells were injected into the dorsal aorta of stage 17 embryos, collected after 60 h of incubation. Embryo survival was monitored at 7 days of chick embryonic development. The model effect between the injections was not significant (P=0.4453).

bedded tissues were sectioned at a 5 μm thickness and stained with hematoxylin and eosin.

5. Experimental design and statistical analysis

To evaluate chicken embryos as an *in vivo* model of tumorigenesis, GFP-expressing HEK 293 cells were injected into the dorsal aorta of stage 17 chicken embryos (incubated for 60 h), and as a control, GFP-expressing CEFs were transferred into the dorsal aorta of stage 17 chicken embryos. Survival rates of the recipient embryos and localization of HEK 293-derived tumors were monitored during the various developmental stages. Histochemical examination of the tumors in the mediasternum areas of the recipient chicks was conducted. The values of each parameter were subjected to ANOVA using the general linear model (PROC-GLM) in the SAS software. When the model effect was significant, the values for each treatment group were subsequently compared using the least-squares method. Differences were considered statistically significant at $P < 0.05$.

RESULTS

1. Detection of GFP-expressing HEK 293 cells in chick embryos

No hemorrhage or leakage of the injected donor cells occurred after injection into the blood vessel. The viabilities of the recipient embryos after transferring the HEK 293 cells and CEFs were 57.1% and 42.9%, respectively (Fig. 1). The injected donor HEK 293 cells showed migration into primarily the brain, eye, heart, kidney and intestine on day 7 (Fig. 2A). In contrast, the migration activity of CEFs

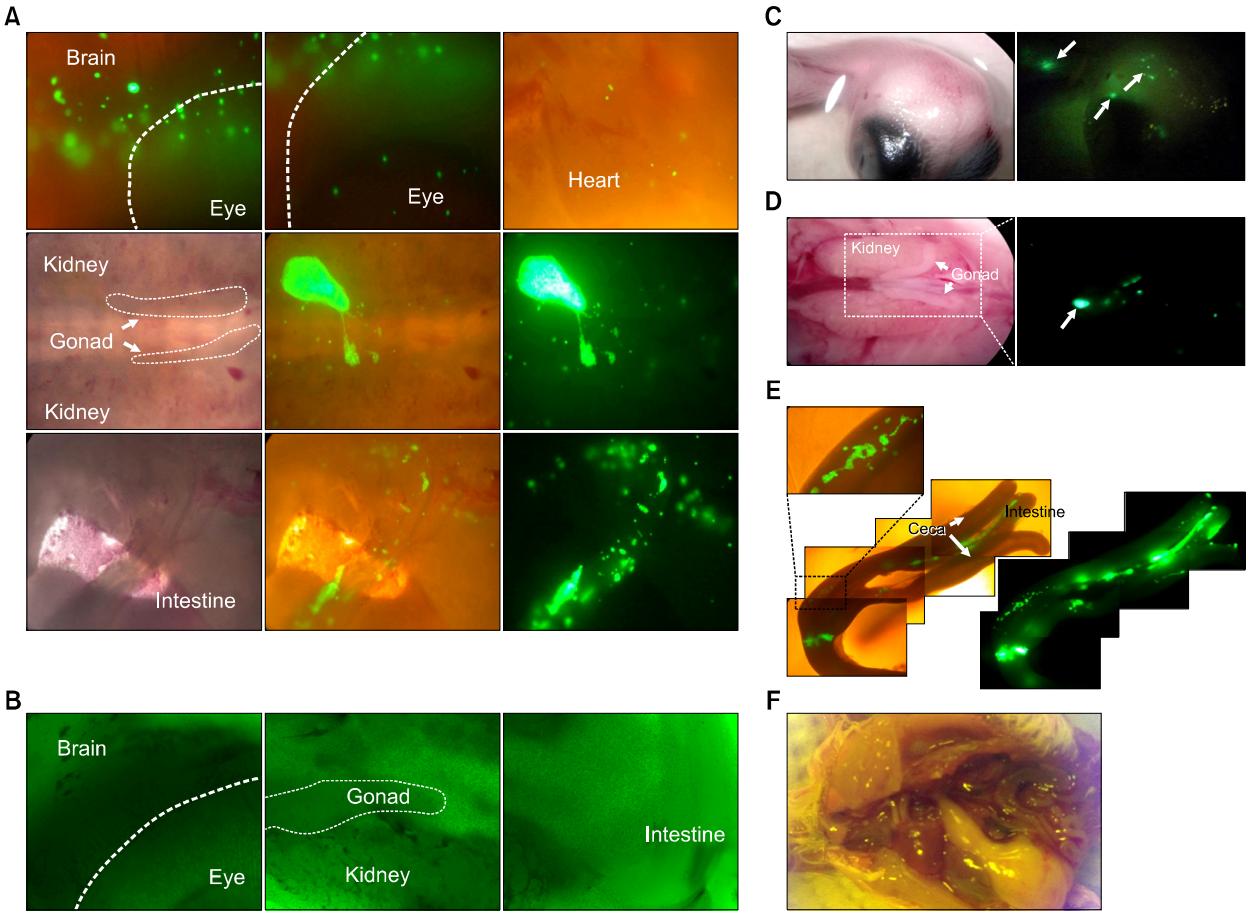

Fig. 2. Localization of (A) HEK 293 GFP-cells and (B) GFP-transfected CEFs after being injected into the dorsal aorta of developing embryos. GFP-transfected HEK 293 cells were detected in the brain, eye, heart, kidney, gonads, intestine and ceca of the injected embryos (C to E), while there were no positive GFP signals from the CEFs (B and F). (A) and (B) Detection of the donor cells at 7 days, and (C to E) at 14 days.

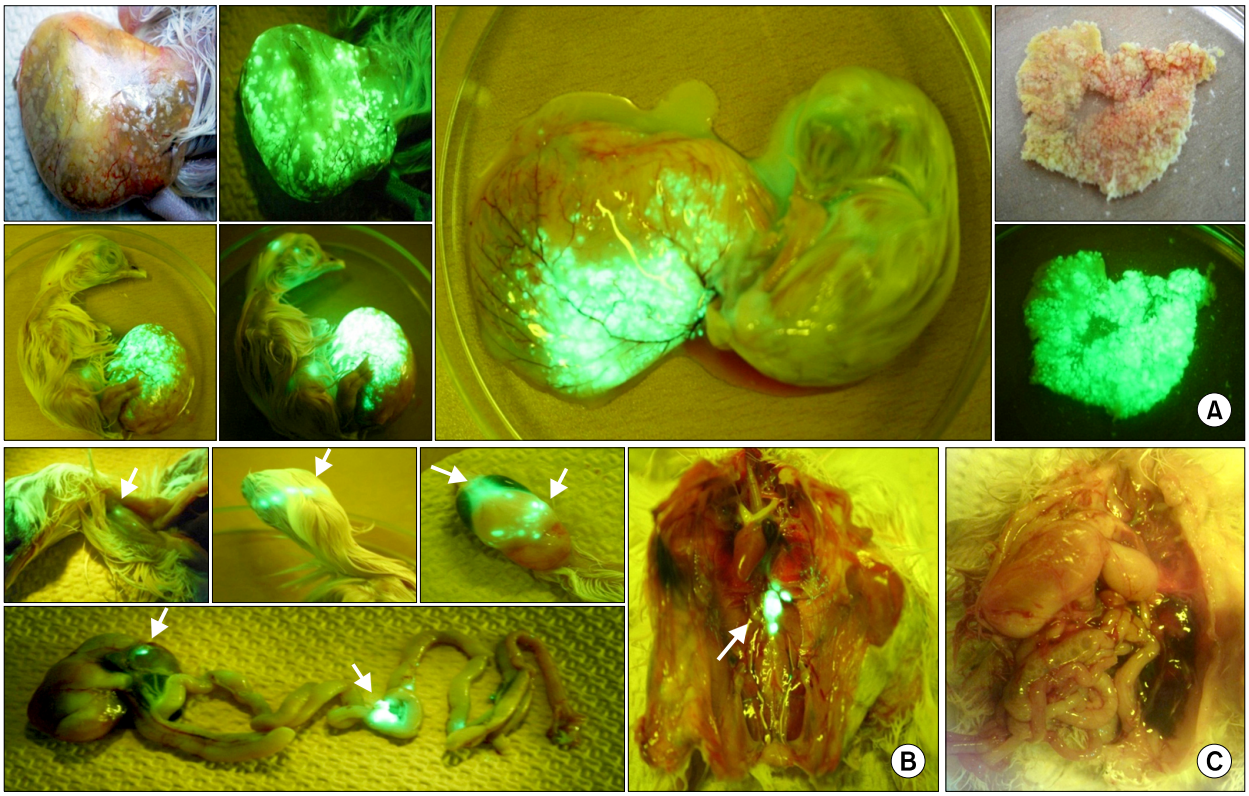

Fig. 3. Autopsy of HEK 293 cell-injected chicks that died before hatching. GFP-expressing HEK 293 cells were injected into the blood vessel of chicken embryos 60 h after incubation. (A) Most tissues in the yolk sac contained HEK 293 cell-derived tissues, and (B) HEK 293 cells (arrows) incorporated into other tissues, including the neck, head, eye, gizzard, intestine and abdominal cavity. (C) GFP CEF-injected chick without a GFP signal.

Table 1. Localization of HEK 293 GFP-transfected cells and GFP-transfected CEFs after injection into the dorsal aorta of chicken embryos

	Day 7			Day 14			Day 21		
	Head	Abdominal cavity	Embryonic muscle	Head	Abdominal cavity	Embryonic muscle	Head	Abdominal cavity	Embryonic muscle
HEK 293 GFP	9/9 (100%)	9/9 (100%)	9/9 (100%)	5/8 (62.5%)	8/8 (100%)	7/8 (87.5%)	5/7 (71.4%)	7/7 (100%)	5/7 (71.4%)
CEF GFP	2/9 (22.2%)	0/9 (0%)	0/9 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
Model effect (P value)	<0.0001	<0.0001	<0.0001	0.0127	<0.0001	<0.0001	0.004	<0.0001	0.040

was less marked than that of HEK 293 cells (Fig. 2B). The injected HEK 293 cells localized as colonized neoplasms as well as single cells (Fig. 2). On day 14 of embryonic development, the localization and distribution patterns of the donor HEK 293 cells were similar to those on day 7 after injection (Figs 2C-E). Interestingly, the GFP-positive colonized neoplasms were much larger (Figs 2D-E). However, in chick embryos injected with GFP-expressing CEFs, GFP-positive cells were generally not detected, except in the heads of two embryos at day 7, where they were sporadically distributed rather than forming colonies.

At the time of hatching, the majority of tumors localized in the yolk sac (Fig. 3A), but many single and colonized GFP-expressing cells were also present in the neck, head, eye, and intestine (Fig. 3B). GFP-positive tumor tissue was found in the abdominal cavity located close to the testis and kidney (Fig. 3B). Colonies of injected HEK 293 cells were detected in all injected embryos between days 7 to 21

(Table 1). GFP-positive colonies were observed in the abdominal cavity of chick embryos at all developmental stages (Table 1). GFP-expressing CEFs were not detected in the recipient embryonic tissues after 7 days (Table 1).

2. Histochemical staining with hematoxylin and eosin after paraffin sectioning

Tumors in the yolk sac of the recipient hatched chick were further investigated by paraffin sectioning and histochemical staining. GFP-expressing tumors in the yolk sac were solid and did not contain cysts or liquid areas (Fig. 4A). To examine GFP-expressing tumor microstructure, paraffin sections were stained with hematoxylin and eosin and showed the typical characteristics of a solid tumor (Fig. 4B).

DISCUSSION

The results of this study indicate that artificial transformation of cells influences their migration activity after injection into developing chicken embryos, while cell transformation itself does not reduce the viability of recipient embryos. A significant increase in colonized neoplasms due to post-injection migration of HEK 293 cells that were artificially transformed was identified, compared with chicken embryonic fibroblasts (CEFs). Although significant tumorigenicity and migration activity were detected after HEK 293 cell transfer, viabilities after transfer of the two cell types were similar, even when cells from a different species (avian and human) were used.

To date, a major concern has been that artificial manipulation of cells for transgenesis reduces post-injection

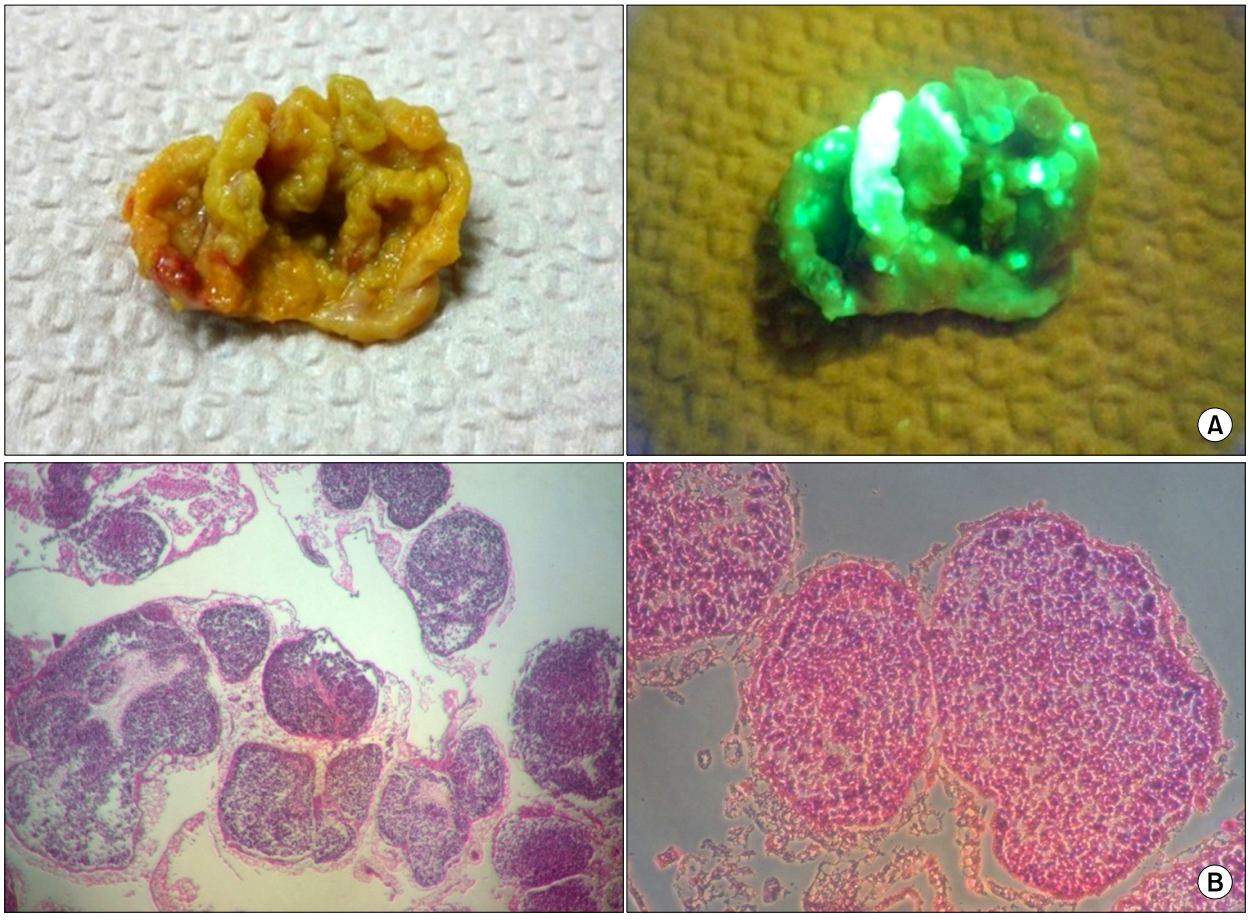

Fig. 4. Tumor tissue in the yolk sac of GFP-positive HEK 293-injected chicks. (A) Strong GFP expression was detected in tumor tissue. (B) Histochemical examination of the mediasternum area of a chick injected with HEK 293 cells, and stained with hematoxylin and eosin (magnification; right panel 100×, left panel 200×).

viability. In this study, HEK 293 cell transfer appeared to result in increased migration, but did not reduce the viability of recipient embryos. In other words, cell transformation before injection may promote migration of the injected cells. HEK 293 cells are not a cancer cell line, rather, they are a transformed cancer-cell-like cell. Their transfers lead to increased tumorigenicity after cell injection. An alternative view is that genetic or cytological manipulation of terminally differentiated cells does not itself negatively affect cell survival, unless tumorigenic activity is not stimulated by the manipulation process or the environmental niche they occupy. These results demonstrate the feasibility of injection of artificially transformed cells into developing chicken embryos for various purposes, including transgenesis and model development. However, careful monitoring of the process to detect tumorigenic activity should be carried out to increase the efficiency and feasibility of development of a new model.

Although physiological and developmental differences exist between humans and birds, avian species-such as chickens and quail-are considered appropriate for investigation of human diseases. The chicken has many advantages as a model animal.[1,6,7] Compared to mammals, the fertilized chicken embryo develops in an egg that is independent of the maternal environment, enabling manipulation of embryos during any developmental stage. The chicken embryo model is regulated by a complex network of processes. The greatest benefit of a chicken embryo model is that the effects of a particular drug treatment can be observed without any external influences.[1,6,7]

Injection system of cancer cells into chick embryos and an in vivo model will enable determination of the external environmental factors and internal processes that regulate, trigger, and can halt tumorigenesis.

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