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In vitro Study of Morphological Changes of the Cultured Otocyst Isolated from the Chick Embryo

Estudio *in vitro* de los Cambios Morfológicos del Otocisto Cultivado Aislado del Embrión de Pollo

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SUMMARY: The aim of this study was to observe morphological changes of the cultured otocysts isolated from various stages of the chick embryo. Isolated otocysts were dissected from embryonic day, E2.5-4.5 of incubation (HH stage 16-26) according to stages of developing inner ear. Morphology of the chick otocyst exhibited an ovoid shape. The width and height of the otocyst were 0.2 mm and 0.3 mm, respectively. Elongation of a tube-like structure, the endolymphatic duct, was found at the dorsal aspect of the otocyst. The cultured otocyst is lined by the otic epithelium and surrounding periotic mesenchymal cells started to migrate outwards the lateral aspect of such epithelium. Notably, the acoustic-vestibular ganglion (AVG) was observed at the ventrolateral aspect of the otocyst. Appearance of AVG *in vitro* can be applied for studying chemical-induced ototoxicity and sensorineural hearing loss. It was concluded that the organ-cultured otocyst of the chick embryo could be used as a model to study sensory organ development of avian inner ear.

KEY WORDS: Chick embryo; Inner ear; Otocyst; Otic development.

INTRODUCTION

Chicken has become a favorable model in developmental biology and stem cell research (Stern, 2005; Intarapat & Stern, 2013). There are several advantages of using chick embryos as the model system: the eggs are available all the year round (Berg *et al*., 1999), the neuroendocrine system is well understood (Ottinger *et al*., 2001), the embryo stages are well-established (Hamburger & Hamilton, 1951), and they are also recommended as a model for testing the toxicants (OECD, 1984; Touart, 2004). Strikingly, chicken was reported to be able to regenerate the new hair cells after exposure to the noise or ototoxic drugs (Cotanche, 1987; Girod *et al*., 1991; Janas *et al*., 1995). A remarkable process of this species brought the researchers to seek for the key factors playing a role in avian hair cell regeneration to overcome this limitation in mammals (Bermingham-McDonogh & Rubel, 2003; Rubel *et al*., 2013).

In mammalian species, the inner ear contains pluripotent stem cells that their regenerative capacity could be induced (Li *et al*., 2003a; Oshima *et al*., 2007). Identification of stem cell sources to generate hair cells *in vitro* for stem cell-based therapy was proposed (Géléoc & Holt, 2014). Previous studies used both ESCs and iPSCs to study auditory organ regeneration and differentiation (Li *et al*., 2003b; Oshima *et al*., 2010; Ouji *et al*., 2012). Cultures of mammalian stem cells with chick embryonic tissues to study hair cell differentiation were reported (Jeon *et al*., 2007; Oshima *et al*., 2010). Cocultures of mammalian stem cells with the chick otocyst and its stromal cells produced hair cell-like cells with stereocilia-like protrusions (Jeon *et al*.; Oshima *et al*., 2010).

The inner ear of the chick embryo contains presumptive sensory hair cells and supporting cells (Sokolowski *et al*., 1993). These cells have ability to replace damaged hair cells (Girod & Rubel, 1991; Bermingham-McDonogh & Rubel; Rubel *et al*.). Because such fascinating process occurs in chicks it is interesting to study embryonic development of sensory organ of this species *in vitro*. Since avian embryos were reported to be able to regenerate their inner ear hair cells after exposure to mechanical and chemical inducers (Cotanche; Girod *et al*.; Janas *et al*.), embryological study of avian inner ear development for medical application is required. Thus the present study aimed to observe morphological changes of developing inner ear using the organ-cultured otocyst of the chick.

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MATERIAL AND METHOD

Chicken fertilized eggs were obtained from Department of Animal Science, Faculty of Agriculture, Kasetsart University. The eggs were cleaned and incubated for 62-108 hrs at 38 °C in a humidified incubator. The embryos reached to HH stage 16-26 (~E2.5-4.5 days of incubation) were staged according to the Hamburger and Hamilton normal stages of chick embryonic development (Hamburger & Hamilton, 1951). The otocysts (otic vesicles) with no periotic mesenchymal tissues were carefully dissected from embryonic day, E2.5-4.5-embryos and then pooled in cold PBS, pH 7.2. Dissected otocysts were observed under stereomicroscope (Olympus, Japan) and their shapes were measured and recorded. Briefly, the size of the otocyst was indicated by width x height. The height of the otocyst was measured by starting from the base at the ventral aspect to the tip of the endolymphatic duct at the dorsal aspect of the otocyst. For organ culture, isolated otocysts were rinsed twice with chick Ringer solution and placed onto a culture dish. Cleaned otocysts were seeded onto 75 cm^2 flask containing Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, GIBCO) supplemented with 10 % Fetal Bovine Serum (FBS, Merck Millipore). Otocyst-culture medium was changed twice a week until the migrating cells had reached confluence. Schematic isolation and culture of the chick otocyst is shown in Fig.1

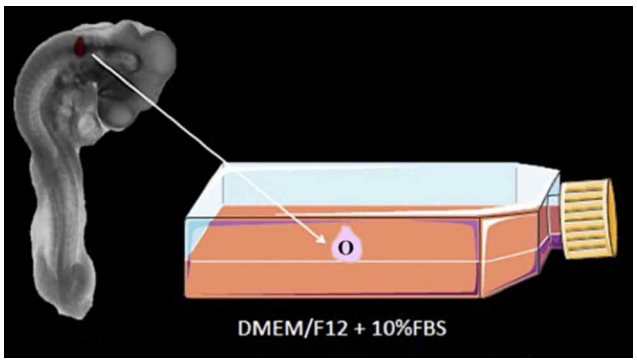

Fig. 1. Schematic of isolation and culture of the chick otocyst (O, otocyst).

RESULTS AND DISCUSSION

Embryonic otocysts were isolated from E2.5-4.5 embryos. We found that E3-otocyst was easily dissected compared to E2.5 and E4.5-otocyts. The size of E2-otocyst was too small to be dissected, while E4.5-otocyst developed more complex structures that had become a hassle to isolate entire otocyst. This suggests that E3-otocyst from HH19 stage chick embryo is suitable for culturing as a whole organ-cultured system. Several problems were described regarding isolation of developing inner ear from early to later stages of otic development in the chick (Honda *et al.*, 2014). However, dispase treatment was suggested to reduce mesenchymal tissue surrounding the otocyst (Honda *et al.*), indicating that this technique is required for isolation of the chick otocyst from the later stages.

Cultured E3-otocyst exhibited an ovoid shape and its size was approximately 0.2 mm in width and 0.3 mm in height (Fig. 2). These characters are similar to mammalian otocyst (Morsli *et al.*, 1998), suggesting a conserved morphogenesis of the otocyst among vertebrates. Elongation of tube-like structure, endolymphatic duct (ED), was observed at the dorsal aspect of the otocyst (Fig. 2); moreover, the ED was first noticed since E2.5 of incubation. Such structure is formed by otic cup closure at the anterodorsal rim of pars superior (Bissonnette & Fekete, 1996; Brigande *et al.*, 2000), giving rise to a endolymphatic sac, a swollen structure located at the end of its duct (Bissonnette & Fekete). Developing ED was well delineated at E3 onwards, indicating the rapid growth of pars superior that can be used to distinguish early and late otocyst (Bissonnette & Fekete). This suggests that appearance of the ED can be used as a landmark of pars superior development that will be useful for studying development of sense organs of equilibrium.

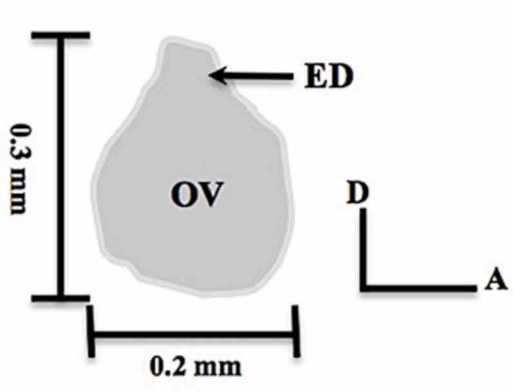

Fig. 2. Morphology of the otic vesicle (OV) or otocyst of embryonic day 3, E3 (HH stage 19) embryo showing the endolymphatic duct (ED) locates at the dorsal aspect of the otocyst (D, dorsal; A, anterior).

In the present study, the chick otocysts were studied since day 2.5 of incubation. At E2.5, we found that developing inner ear can be noticed by thickening of the ectodermal epithelium, otic placode. Invagination of such placode contributed to the otic cup closure and then the otic vesicle is completely formed (Brigande *et al.*; Sai & Ladher,

2015). *In vitro* studies of the key factors that play a role in such processes using the organ-cultured otocyst might help to answer molecular mechanisms underlying otic development *in vivo.*

In culture, adherence of E3-otocyst to the bottom the flask was observed. Furthermore, the otic epithelium (OE) and acoustic-vestibular (AVG) ganglion were also observed (Fig. 3). Obviously, the otocyst is lined by a stratified epithelium and the epithelial invagination can be seen in the middle region of the otocyst (Fig. 3). AVG cells started to delaminate at the ventrolateral aspect of the otocyst and migrating cells were predominantly found at the lateral aspect of the otocyst (Fig. 3). AVG is a cluster of delaminating neuroblasts giving rise to auditory and vestibular neurons (Magariños *et al.*, 2012). The markers for ganglion neuroblast nuclei, Islet-1 and neural processes, TuJ-1 were expressed in the chick AVG (Aburto *et al*., 2012; Magariños *et al*.). These studies indicate specification of sensory neurons in the otocyst.

The majority of migrating cells, periotic mesenchyme, surrounding the E3-otocyst was found this study (Fig. 3). These mesenchymal cells were also found in the mammalian otocyst in which the retinoic acid nuclear receptor genes are expressed (Romand *et al*., 2006). It has been reported that these mesenchymal cells are transformed into bony labyrinth of the inner ear (Lang & Fekete, 2001). Using the organ-cultured otocyst may be useful for identifying a cell lineage of preotic mesenchyme that contributes to the bony part of the inner ear.

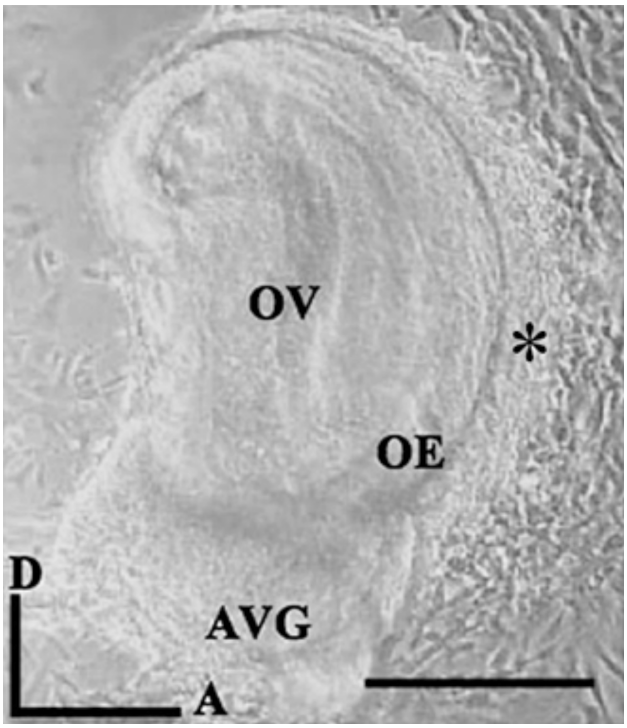

Fig. 3. The chick otocyst isolated from E3 (HH stage 19) embryo (A, anterior; AVG, acoustic-vestibular ganglion; D, dorsal; OE, otic epithelium; OV, otic vesicle or otocyst; asterisk, periotic mesenchyme; Bar, 200 μm)

Heterogeneous population of migrating cells was noticed in the present study (Fig. 4). Two distinct cell types were observed such as neuroblast-like cells and fibroblast-like cells (Fig. 4). Neuroblast-like cells showed small size, prominent nuclei with short processes, whereas fibroblast-like cells exhibited larger size, spindle-shaped with long processes (Fig. 4). Analysis of different types of migrating cells whether they express either Islet-1 or TuJ-1 in the organ-cultured otocyst needs further study. This study provides a basic knowledge of inner ear development of aves that will enable us to propose *in vitro* organ engineering for hearing loss therapy.

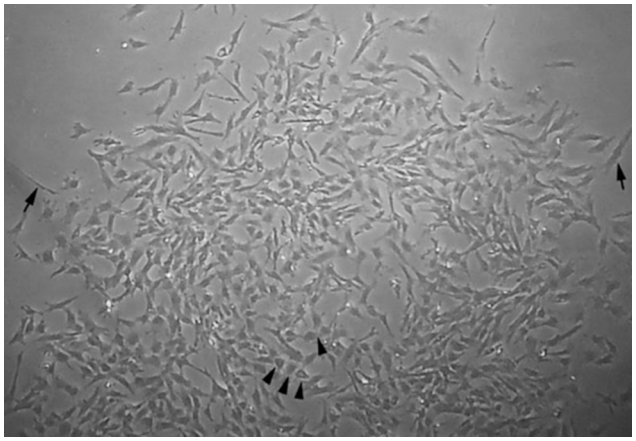

Fig. 4. Photomicrograph of the migrating cells of the otocyst isolated from E3 (HH stage 19) embryo showing neuroblast-like cells (arrowheads) and fibroblast-like cells (arrows)

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INTARAPAT, S.; GONMANEE, T. & THONABULSOMBAT C. Estudio *in vitro* de los cambios morfológicos del otocisto cultivado aislado del embrión de pollo. *Int. J. Morphol., 35(1)*:208-211, 2017.

RESUMEN: El objetivo de este estudio fue observar los cambios morfológicos de otocistos cultivados aislados en las diversas etapas del desarrollo del embrión de pollo. Otocistos aislados fueron obtenidos de embriones día, E2.5-4.5 de incubación (HH etapa 16-26) de acuerdo a las etapas de desarrollo del oído interno. El otocisto de pollo presentó una morfología ovoide. El

ancho y la altura del otocisto fue de 0,2 mm y 0,3 mm, respectivamente. En la cara dorsal del otocisto se visualizó el alargamiento de una estructura similar a un tubo, el conducto endolinfático. El otocisto cultivado está revestido por epitelio ótico y células mesenquimatosas perióticas que comienzan a migrar hacia el exterior de la cara lateral en búsqueda del epitelio. En particular, el ganglio acústico-vestibular (GAV) fue observado en la parte ventrolateral del otocisto. La aparición de GAV *in vitro* puede ser aplicado para el estudio de la ototoxicidad inducida por productos químicos y la pérdida de audición neurosensorial. Se concluyó que el otocisto cultivado de embrión de pollo podría ser utilizado como un modelo para estudiar el desarrollo de órganos sensoriales del oído interno aviar.

PALABRAS CLAVE: Embrión de pollo; Oído interno; Otocisto; Desarrollo ótico.

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