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Ectopic liver and gallbladder in a cloned dog: Possible nonheritable anomaly

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ABSTRACT

Ectopic liver and gallbladder are rare anomalies usually not accompanied by any symptoms and are found during surgical exploration or autopsy. We aimed to find a cause of this anomaly using somatic cell nuclear transfer (SCNT) technology, which can produce genetically identical organisms. A cloned beagle having ectopic organs was produced and died on the day of birth. Major and ectopic organs were fixed and underwent histologic analysis. SCNT was performed using cells derived from the dead puppy to produce reclones. Normality of internal organs in the original donor dog and recloned dogs was evaluated by computed tomography. While a liver without the gallbladder was located in the abdominal cavity of the cloned dog, a well-defined, reddish brown mass with a small sac was also positioned outside of the thoracic cavity. Histologically, they presented as normal liver and gallbladder. Five reclones were produced, and computed tomography results revealed that the original donor dog and reclones had normal liver and gallbladder structure and location. This is the first report of both ectopic liver and gallbladder in an organism and investigation on the etiology of these abnormalities. Normal organ structure and position in the original donor dog and reclones suggests that the ectopic liver and gallbladder is a possible nonheritable anomaly.

1. Introduction

The term “choristoma” *originally* comes from the German word “to separate” and was introduced by Eugen Albrecht in 1904 to refer to a mass of normal tissue located at a site away from its usual location [1]. Choristoma occurring with hepatic or gallbladder tissue is commonly called ectopic or heterotopic liver or gallbladder. Ectopic liver and gallbladder have been recorded since the 19th century [2], but only less than 80 cases of ectopic liver were documented in a recent literature review [3] with lower than 0.5% [4] and 0.4% [5] incidences. Ectopic liver has been found in various sites including the gallbladder [3,6,7], spleen [8], pancreas [9], stomach [10], heart [11], lung [12] and suprahepatic inferior vena cava [13,14], and ectopic gallbladder has been described in the intrahepatic [15] and suprahepatic region [16], and left hepatic lobe [17,18]. Both anomalies are usually asymptomatic, but rare symptoms such as intra-abdominal bleeding due to ectopic liver [10] or epigastric pain caused by ectopic gallbladder [17] have been reported. Lack of symptoms usually results in discovery of these anomalies only during peritoneoscopy,

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laparotomy, or autopsy. Also, almost all the documented cases of these anomalies have been described in human beings, not in animals [1–6,8–12,15–21]. In dogs, ectopic hepatocytes were firstly reported in 2005, in which a bearded collie had a firm and nonpainful mass in the left midabdominal region [22]. Consequently, scarcity of the anomaly, lack of symptoms, and absence of animal models have made it hard to define a cause of ectopic liver and gallbladder.

Somatic cell nuclear transfer (SCNT) is a process for producing genetically identical organisms asexually [23] which includes nuclear removal from a donor oocyte, donor cell injection into the empty perivitelline space, fusion between the cytoplast–cell couplet, and activation of the reconstructed embryo. Since the first cloned animal, Dolly the sheep, was produced using SCNT in 1997 [24], more than 16 mammalian species including mice [25], rats [26], cattle [27], pigs [28], cats [29], and dogs [30] have been successfully produced. Among these, dogs have advantages as animal models for human diseases because of the similarities in their size, longevity, and physiology to humans. Of the nearly 648 known hereditary diseases described in dogs, more than half (352) can be potential models for human diseases (http://omia.angis.org.au, January 2014). A human disease model dog can be generated by replacing a donor oocyte's nucleus with a cell derived from a dog naturally having a heritable disease or with a cell containing a genetically modified transgene [31,32]. For example, a cloned puppy derived from a donor dog having hip dysplasia, which is an inherited disease characterized by hip subluxation and laxity [33,34], also showed signs of hip dysplasia. Because of the identical genetic traits between donor and cloned dogs, SCNT can be used as a tool for studying potential causes of unidentified disease to determine whether it is caused by a genetic modification or not.

In the present study, we report for the first time an occurrence of both ectopic liver and gallbladder in a cloned dog and aimed to investigate their etiology by recloning using cells derived from the clone.

2. Materials and methods

2.1. Animal use

Animal experiments were done following a standard procedure established by the Committee for Accreditation of Laboratory Animal Care and the Guideline for the Care and Use of Laboratory Animals of Seoul National University (approval number is SNU-121130-1).

2.2. Production of a cloned dog

Ear skin tissue from a 10-year-old male beagle (Fig. 1A) was collected and transferred to the laboratory aseptically. The tissue was minced and cultured with Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v:v) fetal bovine serum (Invitrogen). Donor cells were cultured to confluence and retrieved as single cells by trypsinization just after recovery of *in vivo*–matured oocytes.

Recovery of *in vivo*–matured oocytes, SCNT, and embryo transfer were done on the basis of a previous report [30,35]. In brief, surgical oviduct flushing was performed 72 hours after serum progesterone reached 5 to 10 μg/mL [36], and cumulus cells of matured oocytes were removed by repeated pipetting in 0.1% (wt/vol) hyaluronidase in TCM-199. Denuded oocytes underwent enucleation in TCM-199 containing 5 μg/mL of cytochalasin B and 5 μg/mL of bisbenzimide. After injection of a donor cell into the enucleated oocyte, the oocyte–cell couplet was fused with electrical stimulation (2 pulses of 72 V for 15 μs) and activated with 10-μM calcium ionophore and 1.9-mM 6-dimethylaminopurine. Then, the cloned embryos were surgically transferred into one oviduct of a naturally synchronized recipient dog. Pregnancy diagnosis was performed at least 28 days after the embryo transfer by ultrasonography, and serum progesterone concentration, rectal temperature, and fetal heartbeat were monitored for safe delivery [37].

2.3. Recloning and microsatellite (MS) analysis

Skin tissue was collected aseptically from a neonatal dog having ectopic organs on the day of birth, and fibroblasts were acquired by the same methods used for the original donor dog. Recloning also followed the same methods described for production of a cloned dog.

Parentage analysis was performed on the original donor dog, the cloned dog, and recloned dogs to confirm their genetic identity. Eight MS markers including PEZ1, PEZ3, PEZ5, PEZ8, PEZ2, FHC2010, FHC2054, and FHC2079 [38] were chosen for analysis. The isolated genomic DNA samples were dissolved in 50-μL TE (10 mM Tris, 1 mM EDTA, pH 8.0), and length variations were assayed by polymerase chain reaction amplification with fluorescently labeled (FAM, HEX, and NED) locus-specific primers and PAGE on an automated DNA sequencer (ABI 373; Applied Biosystems, Foster City, CA, USA). Proprietary software (GeneScan and Genotyper; Applied Biosystems) was used to estimate the polymerase chain reaction product size in nucleotides.

2.4. Histologic analysis

Organ samples including the heart, lung, spleen, kidney, livers in normal location (mother liver) and ectopic site (ectopic liver), and gallbladder of the cloned dog were fixed in 4% paraformaldehyde until analyzed. After slicing the samples with similar thickness for further processing, the sliced pieces were dehydrated. Subsequently, the pieces were embedded in paraffin wax and cut into 5-μm-thick sections using a microtome. The sections were stained with hematoxylin and eosin and examined under light microscopy to assess histologic normality of the major and ectopic organs.

2.5. Computed tomography

The computed tomography (CT) was performed on the original donor dog (aged 11 years) and recloned dogs (aged 1 year) to compare normality of their internal