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Evidence for the Presence of the Fifth Pharyngeal Arch During the Chick Development

Evidencia de la Presencia del Quinto Arco Faríngeo Durante el Desarrollo del Polluelo

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SUMMARY: The pharyngeal arch (PA) is a transient structure of vertebrate embryos. Their number varies from species to species; nine in lampreys, seven in teleosts, and six in amniotes. Each pharyngeal arch contains a nerve and an artery as well as skeletomuscular elements. There has been a long-standing controversy about the formation of the fifth PA. Here, we investigated the formation of the fifth PA by tracking the distribution of neural crest cells in pharyngeal arches in chick embryos. Fertilized chick and quail eggs were used. Neural crest cells were detected by using quail-chick cell tracing technique and *in ovo* electroporation of EGFP constructs. Our results showed that neural crest cells from the post-otic level contributed to all post-otic pharyngeal arches (PA3-6). In contrast, neural crest cells from the somite 1-3 level avoided to invade the third pharyngeal arch, although they gave rise post-otic pharyngeal arches. Based on the trajectory of neural crest cells, the fifth pharyngeal arch was visible between the fourth and sixth pharyngeal arch, although it was considerably smaller compared to other arches. The fifth PA forms during early development at least in avian species. It is closer to the sixth PA than the fourth PA. This is in line with the formation of the fifth PAA, which can form a bypass and even fuse with the sixth PAA.

KEY WORDS: Fifth pharyngeal arch; Neural crest; Chick embryos; Quail chick chimera; *In ovo*-electroporation.

INTRODUCTION

Pharyngeal segmentation is a defining feature of vertebrate embryos. It is first evident as a series of bulges on the lateral surface of the embryonic head during development, in structures called the pharyngeal arches (PAs) (Lumsden *et al*., 1991; Graham & Richardson, 2012). The anterior and posterior boundary of the PAs are defined by the pharyngeal pouch and cleft (Schilling & Kimmel, 1994; Horigome *et al*., 1999). The PAs contribute to the pharyngeal apparatus which supports feeding and respiration (Graham & Richardson, 2012; Frisdal & Trainor, 2014).

Organization and development of the PAs is highly conserved and involves interplay between a number of disparate embryonic tissues derived from the three primary germ layers, the ectoderm, mesoderm and endoderm (Graham & Smith, 2001; Graham & Richardson, 2012; Shone & Graham, 2014). Externally, the ectoderm gives rise to the epidermis and forms neurogenic placodes, which contribute to sensory neurons. The endoderm, which lies internally, forms the lining of the pharynx, as well as a number of special organs, such as the thyroid, parathyroids and thymus. Cells of the mesoderm and the neural crest fill the PAs and become located between the ectoderm and endoderm. The mesoderm, which lies centrally within the arch, forms endothelial and skeletal myogenic cells. Neural crest cells (NCCs) that surround the mesodermal core contribute to the skeletal, connective and smooth muscular tissue (Noden, 1983; Lumsden *et al*., 1991; Graham & Smith, 2001; Graham & Richardson, 2012). Moreover, NCCs play also roles in directing patterning formation of PA derivatives (Wagner, 1949; Noden, 1983, 1984; Graham & Richardson, 2012).

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In vertebrates, the PAs number varies from species to species. While two constant PAs appear anterior to the otic level, a variable number of the post-otic PAs emerge in lampreys (seven PAs), teleosts (five PAs) and amniotes (three PAs) (Schilling & Kimmel, 1994; Horigome *et al*., 1999). In the aquatic species (lampreys and teleosts), the numbers of the post-otic PAs is higher than in the tetrapods (amniotes), since they form the gills which serve as breathing organs. The first PA (mandibular arch) contributes mainly the skeletomuscular tissue of the upper and lower jaw. The second PA (hyoid arch) gives rise mainly to the upper part of the hyoid bone and the facial muscles. The post-otic PAs form the branchial apparatus in teleosts. In amniotes, the third PA participates in the formation of the lower part of the hyoid bone and the stylopharyngeus muscle. The fourth PA contributes to the laryngeal cartilage and muscles as well as the pharyngeal muscles. The sixth PA is believed to participate in the generation of the larynx (Goodrich, 1930; Graham & Richardson, 2012; Frisdal & Trainor, 2014; Poopalasundaram *et al*., 2019). However, embryologists have debated whether the fifth PA forms or not for almost two hundred years. It is believed to disappear soon after it forms (Gray, 1918; Shone *et al*., 2016).

Each PA contains an artery, the pharyngeal arch artery (PAA) which connect the heart to the dorsal aorta (Lehmann, 1905). Thus, there should be six pairs of PAAs during the development of amniotes. The initially symmetrical PAA undergoes asymmetric remodeling to form the definitive blood vessels in adults. The first two PAAs disappear to a large extent. The third PAA develops into the carotid artery. The fourth PAA forms the subclavian artery and the aortic arch. The sixth PA gives rise to pulmonary artery (Frisdal & Trainor, 2014). The fifth PAA is believed to be missing or vestigial in amniotes. In chick embryos, the fifth PAA in form of vessels branching from the sixth PAA was observed between HH20 (Hamburger-Hamilton-stage) and HH25 by scanning electron microscopy (Hiruma & Hirakow, 1995). It implies that the fifth PA may arise during these stages in the chick embryo.

In this study, we investigate the formation of the fifth PA by tracking NCCs migration into the PAs (Rinon *et al*., 2007). NCCs were detected by using quail-chick tissue grafting and *in ovo* electroporation of EGFP (enhanced green fluorescent protein) constructs. We found that NCCs from the post-otic level contribute to the all post-otic pharyngeal arches, while NCCs from the somite 1-3 level give rise only to the PA4-6. Based on the streams of NCCs, the PA5 was evident between PA4 and PA6, although it is much smaller than the other PAs.

MATERIAL AND METHOD

Avian model embryos. Fertilized chick and quail eggs were obtained from the Institute for Animal Science at the University of Bonn. The eggs were incubated at a temperature of 37.8 °C and at 80 % relative humidity until the required stages. The developing stages were determined according to the staging system of Hamburger and Hamilton and were indicated as HH (Hamburger & Hamilton, 1992).

Grafting of the neural tube half. To investigate the contribution of the neural crest to specific pharyngeal arches and whether the fifth PA is formed during the early development, homotypic unilateral transplantation of the quail neural tube into a chick embryo at HH 10 at the first somite level was carried out in this study. Details of the grafting procedure were described in our previous studies (Bai *et al*., 2016). The host embryos were reincubated for 2 days. The chimera embryos were rinsed in phosphate-buffered saline (PBS) and then fixed overnight in 4 % paraformaldehyde at 4 °C. Quail cells were detected by an anti-quail antibody (QCPN) (dilution 1:100; Developmental Studies Hybridoma Bank, Iowa City, IA) as a primary antibody. Nitroblue tetrazolium (NBT) and X-phosphate (Boehringer Mannheim) were used as chromogens to reveal a blue signal (viewed by black spots). The QCPN expression was examined under the Nikon SM21500 microscope.

***In ovo*-Electroporation.** To improve labelled the NCCs tracing, the neural crest at axial levels of the post-otic and cranial somite level at HH10 in chick embryos were transfected through focal electroporation of EGFP constructs. After windowing the egg, the embryo was visualized by injecting 10 % black India ink (Pelikan Fount; PLK 51822A143) in PBS solution under the blastoderm. For the electroporation, the Locke-solution was used to facilitate manipulation in the embryo, and the manipulation was carried out under a dissecting microscope (Leica). To visualize the plasmid solution, it was mixed with 0.2 % Fast Green (Sigma) (Krull, 2004; Scaal *et al*., 2004). The plasmid-Fast Green solution (7-9µg /µl, final concentration of plasmids) was sucked into very fine glass capillaries. The glass capillary was then inserted into the neural tube of HH10 stage embryos and a small drop of the solution was injected using air pressure at the levels of the post-otic region and cranial somites. Immediately after the injection, the targeted part of the embryo was electroporated. Two electrodes were prepared for electroporation (electrode length/diameter: 1.5-2.0/0.2 mm), the positive electrode was placed dorsal to the embryo, while the negative electrode was placed ventral to the embryo. The electrodes were placed parallel to each other leaving the embryo in the middle. Uncontrolled spreading of the electric field was prevented by using nail polish on

the non-opposing surfaces of the electrodes. Due to its negative charge, the DNA molecule was attracted towards the anode. In this experiment, we used high/low voltage mode, 70/7 volts, 0.01/ 99.99 ms (pulse length, intervals between high and low voltage pulses) for high voltage, 2 cycles of low voltage pulses with 50ms/ 200ms (pulse length, interval between two pulses). After electroporation, eggs were re-incubated. After 2 days of reincubation, EGFP expression was examined under the Nikon SM21500 fluorescence microscope with an EGFP filter.

RESULTS

The pharyngeal segments form first anteriorly and then subsequently posteriorly. The time table for the formation of the pharyngeal arch (PA) begins at HH13 when the first two pharyngeal pouches in chick are visible (Veitch *et al*., 1999). The first and second PA were distinct at HH14, and subsequently the PA3 arises at HH15. By HH19, the PA4 was apparent. The last segment, PA6 had completely formed posterior to the fourth pouch at HH22 (Hamburger & Hamilton, 1992).

To investigate the question of whether the fifth PA (PA5) had arisen during the early development, we first analyzed the morphology of the PAs. The lateral view of a chick embryo at HH22 showed all the PAs formed at this stage (Fig. 1). PA1, 2, 3, 4 and 6 arranged in line from cranial to the thorax wall. At this stage the heart and thus also the thorax was located still anterior to the forelimb bud. The last PA, which can be distinguished from the surrounding structure through the different tissue density, was fused to the anterior boundary of the thorax wall. Between the PA4 and 6, however, no PA5 could be recognized.

Since the PA5 cannot be identified through the surface relief, we analyzed the distribution of NCCs, which could provide contour of PAs. It has been shown that NCCs are located beneath the surface ectoderm and surround the myogenic core of the PAs (Rinon *et al*., 2007). Thus, NCC may sketch out the contour of the PAs. According to the observation made by Ferguson & Graham (2004), NCCs from post otic vesicle and cranial somites level contribute to the pharyngeal arches 3-6. To visualize the caudal PAs, both grafting and *in ovo* electroporation were carried out to trace NCCs in PAs.

By the grafting experiments, the neural tube half at the first somite level was homotopically transplanted from a quail to a chick embryo at HH9 just prior to NCCs migration. The host embryos were reincubated until stage HH22, and quail cells were detected with the QCPN antibody, which specifically stains a nuclear epitope of quail cells (Selleck & Bronner-Fraser, 1995; Wilting *et al*., 1995). Similar to the previous observation using the DiI cell tracing method, NCCs from the graft, viewed by black spots, migrated into the PA4 and 6. However, the PA5 was still not recognized (Fig. 2, n=3). This could have been due to the low resolution of this cell tracing technique.

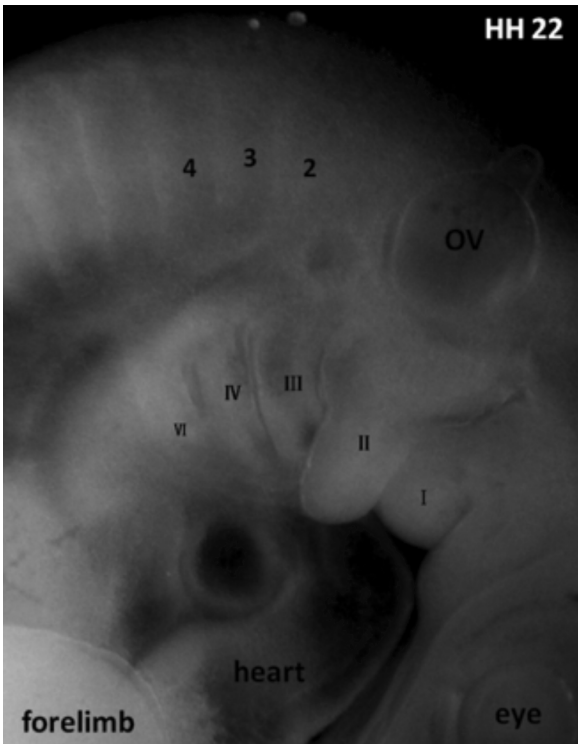

Fig. 1 Lateral view of a chick embryo of HH22 stage when all pharyngeal arches (I, II, III, IV and VI) have formed. The pharyngeal arch V is not visible. OV: otic vesicle; 2/3/4: Somite 2, 3, 4.

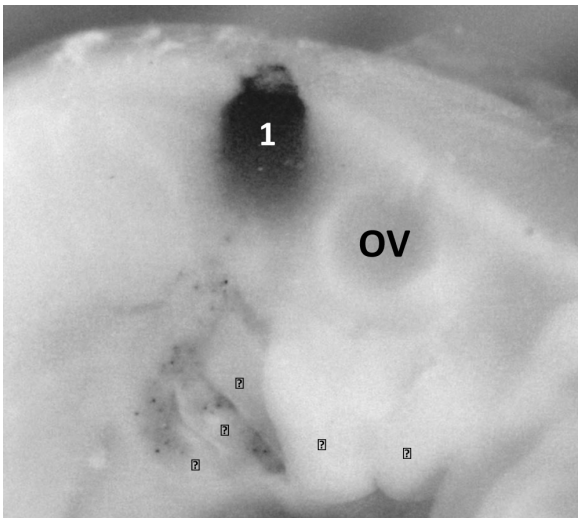

Fig. 2 The quail-chick chimeric embryo with whole-mount immunohistochemistry using QCPN at the HH22 stage. The black labelled structure at the somite 1 (**1**) level is the neural tube half grafted from a quail embryo. Neural crest cells (their nuclei were indicated by spots) from the graft migrated into the pharyngeal arches IV and VI. The pharyngeal arch V is not recognized. OV: otic vesicle.

To improve the cellular resolution, we labelled NCCs through focal electroporation of EGFP constructs into the neural crest at the axial level of the post-otic and cranial somites at HH10. Embryos were re-incubated until HH22 (day 4) after electroporation. Though we tried to restrict the transfection to one somite scope using high viscosity of the EGFP solution (7-9μg /μl), the drop of EGFP solution injected into the center canal of the neural tube spread out. Thus, EGFP-positive neural crest was located at the level of about two somites. After injection and electroporation at the post-otic level, EGFP-positive NCCs were formed at the post-otic and first somite level. They migrated into PA3-6 (Fig. 3A and A', n=4). As supposed, the distribution of EGFP-positive NCCs provided very clear contour of the PAs. The PA3 was the largest one among all the PAs labelled by EGFP. The PA4 was much smaller than the PA3. The cleft between these two arches was broad. Though the PA6 was fused to the thorax wall, as seen in the morphology (Fig. 1), the caudal most distribution of the NCCs clearly outlined its typical pharyngeal arch form. Though the PA6 was little shorter than the PA4, their width was very similar. Between them, a very narrow structure was visible. This segment had a broad anterior cleft and a very narrow posterior cleft. Thus, this segment was separated by very clear boundaries from its anterior and posterior neighbour structures, and classified as PA5.

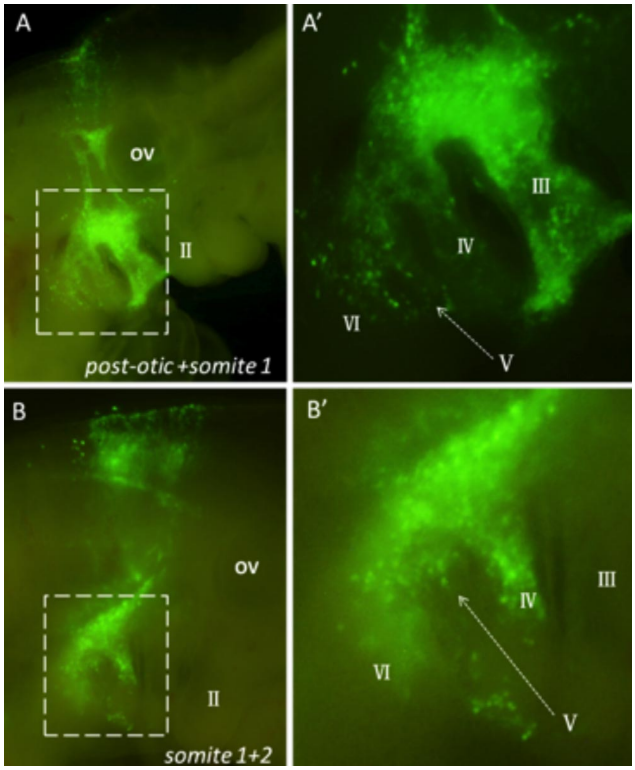

Fig. 3 Trajectory of neural crest cells (viewed by EGFP) in pharyngeal arches reveals the formation of the pharyngeal arch V. A: NCCs streams from the post-otic and the first somite level, respectively. B: NCCs from the level of somite 1+2. A' and B': Magnification of the white box region in A and B. II, III, IV, V, VI: pharyngeal arches; OV: otic vesicle.

The major difference to the contribution of neural crest from the post-otic and somite 1 level was that NCCs from somite 1+2 level did not contribute to the PA3. They were located only in the PA4-6 (Fig. 3B and B', n=5). The size and form of PA4 and 6 outlined through these NCCs were very similar between these two different levels of NCC origins. In this series, we observed also a very narrow segment viewed by EGFP-positive cells, which can be referred to as the PA5. Similar results were observed in the series with EGFP-traced NCC from the somite 2 and somite 2+3 level (data not shown).

DISCUSSION

The pharyngeal arch forms transiently during vertebrate embryogenesis. Its number varies from species to species, for instance, nine in lampreys, seven in teleosts, and six in amniotes (Schilling & Kimmel, 1994; Horigome *et al*., 1999). Among the six PAs, the fifth PA is a controversial one, since it is difficult to see during the embryonic development, although the fifth pharyngeal arch artery has been shown in chicken and mammalian (Reagan, 1912; Hiruma & Hirakow, 1995; Gupta *et al*., 2015). In this study, we observed the small but distinguishable fifth PA in chick embryos by visualizing post-otic PAs with the help of EGFP-expressing NCCs.

Organization and development of the PAs is highly conserved. Each PA is separated from neighbouring PA by a cleft in the outer surface and a pouch in the inner surface and has the same architecture, which is composed of two epithelia, the endoderm and ectoderm that together line a mesenchymal core composed of both mesoderm and NCCs in vertebrate embryos (Graham & Smith, 2001; Graham *et al*., 2005; Graham & Richardson, 2012; Frisdal & Trainor, 2014; Shone & Graham, 2014). NCCs are a population of cells that delaminate from the neural crest via an epithelial to mesenchymal transformation, and migrate in discrete segregated streams, filling each PA (Le Douarin, 1990; Villanueva *et al*., 2002). Since NCCs are located just beneath the surface ectoderm (Graham & Smith, 2001; Rinon *et al*., 2007; Graham & Richardson, 2012), the visualization of NCCs can reveal the contour of the PAs. While the preotic NCCs colonize the first PAs, the post-otic NCCs populate the posterior PAs. They initiate the migration in the dorsolateral pathway (between the ectoderm and the dermomyotome) at HH10. By HH13, NCCs stop migrating dorso laterally and begin to migrate in the ventral pathway up until HH21 (Reedy *et al*., 1998; Kuo & Erickson, 2011). Thus, the EGFP-expressing NCCs by electroporation at the post-otic level can reveal the PA3-6.

Each PA contains a pharyngeal arch artery (PAA) which is primitive aortic arch between the ventral and the dorsal aorta. The PAAs form in concert with the PAs in a cranio-caudal gradient (Hiruma & Hirakow, 1995; Frisdal & Trainor, 2014). In developing chick embryos, the first PAA is found to form at HH12. The second PAA was found at HH14 and the third PAA at HH15. By HHl9, the first PAA has disappeared when the fourth PAA appears. The fifth and sixth PAA are observed as capillary plexus at HH20 and HH21. The fifth PAA forms a bypass with the sixth PAA during HH22 to HH25 (Hiruma & Hirakow, 1995). In accordance with the spatially and temporally correlated development of the PAAs and PAs, the formation of PA5 visualized in this study is highly consistent with the formation of the fifth PAA in the spatial and temporal manner.

The neural crest from the post-otic to the somite 3 level gives rise to derivatives of the post-otic PAs, thereby participating in septation of the outflow tract of the heart (Kirby *et al*., 1983; Kirby, 1987; Phillips *et al*., 1987; Kirby & Waldo, 1990; Ali *et al*., 2003; Kirby & Hutson, 2010). However, the neural crest at the post-otic level differs from those at the somite level. After labelling with DiI at the post-otic level and the level of somites 1-3, Ferguson & Graham (2004), observed that NCCs contribute to all the post-otic PAs. In this study, we observed different contribution between NCCs from the post-otic and somite level; while the post-otic NCCs populate all post-otic PAs, NCCs from the level of somite 1-3 avoid the third PA. In other words, the third PA obtains only NCCs from the post-otic level. In contrast, the PA 4-6 receives NCCs from both the post-otic and somite level (Fig. 4).

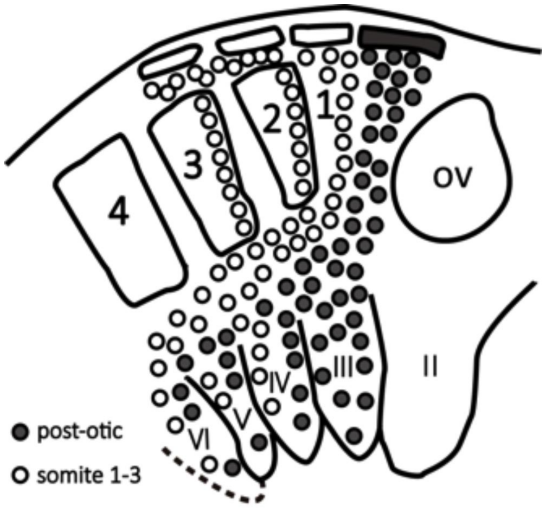

Fig. 4 Schematic illustration of the results. NCCs from the post-otic level (dark spots) contribute to the pharyngeal arches III-VI, while NCCs from the somite 1-3 level (open circles) give rise only to the fourth, fifth and sixth pharyngeal arches (IV, V and VI). 1/2/3/4: Somite 1, 2, 3, 4; OV: otic vesicle.

Taken together, the fifth PA forms during early development at least in avian species. It is closer to the sixth PA than the fourth PA. This is in line with the formation of the fifth PAA, which can form a bypass and even fuse with the sixth PAA. In mammals and human, the fifth PAA can persist after birth (Gupta *et al*., 2015).

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WANG, J.; LIANGA, J.; WANG, Y.; TAO, H.; PU, Q.; KUERTEN, S.; TIEMANN, I.; TEIS, S.; PATEL, K. & HUANG, R. Evidencia de la presencia del quinto arco faríngeo durante el desarrollo del polluelo. *Int. J. Morphol., 42(5)*:1322-1327, 2024.

RESUMEN: El arco faríngeo (AF) es una estructura transitoria de los embriones de vertebrados. Su número varía de una especie a otra; nueve en lampreas, siete en teleósteos y seis en amniotas. Cada arco faríngeo contiene un nervio y una arteria, así como elementos esqueleto musculares. Ha existido una larga controversia sobre laformación del quinto arco faríngeo. En este trabajo investigamos la formación del quinto AF mediante el seguimiento de la distribución de las células de la cresta neural en los arcos faríngeos en embriones de pollo. Se utilizaron huevos de pollito y codorniz fertilizados. Las células de la cresta neural se detectaron mediante la técnica de rastreo de células de polluelos de codorniz y la electroporación *in ovo* de construcciones de EGFP. Nuestros resultados mostraron que las células de la cresta neural del nivel postótico contribuyeron a todos los arcos faríngeos postóticos (AF3-6). Por el contrario, las células de la cresta neural del nivel somita 1-3 evitaron invadir el tercer arco faríngeo, aunque dieron lugar a arcos faríngeos postóticos. Según la trayectoria de las células de la cresta neural, el quinto arco faríngeo era visible entre el cuarto y el sexto arco faríngeo, aunque era considerablemente más pequeño en comparación con otros arcos. El quinto AF se forma durante el desarrollo temprano, al menos en especies de aves. Se encuentra más cerca del sexto AF que del cuarto AF. Esto concuerda con la formación del quinto AAF, que puede formar un bypass e incluso fusionarse con el sexto AAF.

PALABRAS CLAVE: Quinto arco faríngeo; Cresta neural; Embriones de polluelo; Quimera de pollito de codorniz; en ovoelectroporación.

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