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Current genomic editing approaches in avian transgenesis

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ABSTRACT

The chicken was domesticated from Red Jungle Fowl over 8000 years ago and became one of the major food sources worldwide. At present, the poultry industry is one of the largest industrial animal stocks in the world, and its economic scale is expanding significantly with increasing consumption. Additionally, since Aristotle used chicken eggs as a model to provide remarkable insights into how life begins, chickens have been used as invaluable and powerful experimental materials for studying embryo development, immune systems, biomedical processes, and hormonal regulation. Combined with advancements in efficient transgenic technology, avian models have become even more important than would have been expected.

1. Introduction

The chicken has several advantages as a model animal. Due to its oviparity, chicken embryos can be easily accessed and chemically processed to evaluate cellular and molecular differentiation mechanisms. In experimental procedures, chicken embryos can be treated and examined without maternal or external effects; therefore, researchers can expect and detect treatment effects alone in chicken embryos. Thus, chicken embryos provide the best model for investigating hormones and hormone regulatory networks during developmental embryonic stages. For large-scale experimental treatments, the developmental stages of chicken embryos can be easily synchronized without individual variation. In 2004, the chicken genome project was completed and chicken genomic sequences were released to the public domain [15]. Thus, genomic information and the structure of genes and target sites of interest can be readily retrieved from a Web-based database. Currently, the genomic sequences of the zebra finch and the turkey are also publicly accessible on the Internet and evolutionary comparisons among target genes can be made between the different avian species [9,56]. Finally, recent technical advances in avian transgenesis allow efficient extensions of experimental protocols and research areas, which will help provide a more comprehensive understanding of developmental processes and molecular mechanisms.

2. Aves as animal models

An animal model is a living animal that is used to study human diseases as well as for basic research. To date, rodents are the most commonly used animals but they have several limitations such as the difficult access to living mouse fetus and the indispensable maternal influences to mouse fetus [48]. Thus, novel animal models should be developed for accurate and efficient assessments in various species. In particular, chicken and quail have been used in various studies as an alternative animal model. In studies on developmental endocrinology, chicken embryos have been used to examine cellular differentiation and the maturation of endocrine glands, and ontogenic alterations in embryonic glands and their target organs [10]. Gene expression patterns have been analyzed during the functional development of embryonic glands [43], and signaling factors have been treated to verify their effects on ontogenic changes in chicken embryonic glands [2]. These studies demonstrated that the gene expression patterns and ontogeny that were observed in chicken and mouse showed high similarity; therefore, the chicken can serve as an excellent model to validate the genetic and molecular mechanisms underlying gland development.

In hens, the epithelial cells of the oviduct produce large quantities of egg-white protein in daily cycles. Thus, the chicken oviduct system has a considerable advantage as a model system for studying the hormonal regulation of specific gene expression. The chicken oviduct system has been extensively examined *in vivo* and *in vitro* to study hormonal responsiveness and regulation for cell growth, gene expression, and ovarian cancer [20]. To investigate harmoniously orchestrated interactions between steroid hormones and gene expression for egg-white proteins, we developed *in vitro* culturing procedures for chicken oviduct epithelial cells and identified a cascade of events in cultured oviductal cells after hormone induction [20].

In 2010, Sato et al. demonstrated the vascular morphogenetic process using transgenic quail embryos [45]. Transgenic quail that

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had a yellow fluorescent protein (YFP) gene that was controlled by the mouse *Tie1* promoter were generated by lentiviral transduction. Transgenic (*tie1*:H2B-eYFP) quail embryos expressed the reporter YFP in their endothelial cells, and early embryonic vascular morphogenesis was investigated using a time-lapse dynamic imaging system. The study demonstrated that avian transgenic technology represents a powerful approach for addressing the challenge of studying the mechanical, molecular, and cellular mechanisms involved in vascular development [45]. An alternative experimental bird, the zebra finch, is considered a suitable model for studying vocal learning processes. Recently, transgenic songbirds were successfully generated using lentiviral transduction [1]; this offers a great opportunity to develop an alternative genetic model for vocal learning.

3. Strategic approaches in avian transgenesis

To create transgenic birds, various approaches have been tried, essentially starting by adapting techniques that were established in mammals. However, the production of transgenic birds through germline transmission either failed or the transgenesis efficiency was too low because of evolutionary and physiological differences between aves and mammals. Love et al. [25] microinjected plasmid DNA into the germinal disc of fertilized chick embryos. However, the direct microinjection technique is not currently used due to its low efficiency relative to the large number of hens that need to be killed to collect fertilized eggs. *In ovo* electroporation into chicken embryos was an alternative and useful method for introducing transgenesis in specific tissues during early developmental stages [19]. The *in ovo* electroporation technique allowed gene transfers into living embryos to analyze gene function and molecular mechanisms during embryo development. Nevertheless, this technique is only suitable for temporal transgene expression within small windows of developmental stages and can be used with regionally restricted expression in chicken embryos. Producing transgenic offspring via *in ovo* electroporation would be almost impossible.

The most reliable method for generating transgenic chickens is virus-mediated transgenesis, regardless of whether it involves infection into blastoderms or germ cells [32,35,38,44,46]. Salter et al. [44] first applied retrovirus transduction to chicken blastoderms at stage X to produce transgenic chickens. Transgenic quail were produced by injecting a replication-defective pantropic retroviral vector based on Moloney murine leukemia virus (MoMLV) that was pseudotyped with vesicular stomatitis virus G protein (VSV-G) into the quail blastodermal layer [32]. However, in the this study, the reporter expression of the green fluorescent protein (GFP) gene that was controlled by the MoMLV long-terminal repeat (LTR) promoter was not detected because of a transgene silencing effect [32]. Retrovirus- and lentivirus-transduced primordial germ cells (PGCs) have been transplanted into recipient embryos to generate transgenic chickens and quail [35,38,46]. Compared with blastoderm injection protocols in which somatic cells and germ cells are infected by transgene-containing viruses, germ cell-targeted transduction is a more efficient tool because the germ cell is the only germline-transmittable cell type that affects the next generation.

Virus transduction is a versatile and useful protocol but a virus-independent transgene delivery system is necessary due to safety issues related to infectious virus reconstruction in the host. Thus, nonviral transfection into germline-competent cells by lipofection or electroporation would represent the best option for avian transgenesis. PGC is a precursor cell of germ cells during embryo development. In the chicken, PGCs arise from the center area of the epiblast in the blastoderm, termed the area pellucida; they subsequently move into the germinal crescent of an extraembryonic site [50]. At the beginning of blood vessel formation, PGCs migrate to the embryonic genital ridges through blood circulation. These PGCs can be retrieved from different embryonic sites at different stages: the germinal crescent, embryonic blood vessel, and embryonic gonads [18]. Since PGCs finally differentiate into sperm through the spermatogenetic process in male testis or into oocytes through oogenesis in the female ovary after sexual maturation, PGCs are considered a candidate vehicle for delivering transgenes to the next generation (Fig. 1). For germline transmission, Wentworth et al. [57] first characterized and manipulated quail PGCs to produce germline chimeric quial that have both their own (endogenous) germ cells and transferred (exogenous) germ cells. In the chicken, Tajima et al. [51] isolated circulating PGCs from blood vessels of embryos at stages 13–14 and transferred those PGCs into the blood vessels of recipient embryos at stages 14–15. The germline transmission efficiency ranged from 0.0% to 11.8%. As another source, chicken PGCs were isolated from embryonic gonads at 6 days, at which time PGCs had already completed their migration through the blood vessels. Notably, gonadal PGCs (gPGCs) retain the capacity to migrate into genital ridges after introduction into blood vessels in recipient embryos [6]. The manipulation of gPGCs extended the use of PGCs for *in vitro* culture as well as germline chimera production because of the ease of isolation and the relatively large number of PGCs that can be harvested from embryonic gonads (Fig. 1). In the first report of germline chimera production using gPGCs, the efficiency of germline transmission was between 1.3% and 3.1% [6]. Park et al. [39] advanced the short-term culture technique for chicken gPGCs and improved the germline transmission efficiency to 10.7–49.7%.

More recently, a chicken PGC *in vitro* culture system has been established [7,26,55]. Using cultured chicken PGCs that were isolated from embryonic blood vessels, van de Lavoir et al. [55] successfully produced transgenic chickens as well as germline chimeras after genetic modification and transplantation. In the last year, two separate groups reported the combination of chicken PGC culture with transfection of transposon elements, *piggyBac* and Tol2, to enhance stable genomic integration and the expression of transgenes for transgenic production [27,40]. Transposon elements that are mediated by the transposase catalytic process can be used in strategic applications to achieve efficient avian genomic modification. Using *piggyBac*-mediated gene transfer into chicken PGCs, we demonstrated that the efficiency of germline transmission of donor PGCs after *piggyBac* transposition was 95.2% on average, and as was expected, half of the donor-derived offspring (52.2%) were transgenic due to heterozygous transgenes in the donor PGCs [40]. The transposon-mediated approach can be widely adapted to transgenesis in other avian species without running risks such as using viral vectors.

Other germline-competent cells, specifically blastodermal cells, embryonic stem (ES) cells, embryonic germ (EG) cells, and spermatogonial cells, have also been used to transmit manipulated cells into the next generation, but the efficiency of germline transmission is generally too low compared with PGC techniques [21,37,41,42].

4. Specific genetic modifications in the chicken genome

4.1. Overexpression and knockdown

To overexpress specific genes, constitutive and strong promoters have been used in various species, including aves. One strong promoter was isolated from the Rous sarcoma virus (RSV), which was the first oncovirus and one that causes sarcoma in chickens. Since Gorman et al. [13] verified the strong transcriptional activity