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Genome editing in poultry - opportunities and impacts

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ABSTRACT

Poultry products (meat and eggs) are a major source of animal protein on which the world is increasingly reliant to feed a rapidly growing population. Improved breeds and advances in farm management practices have had a large impact on the poultry industry. For example, using current genetic stock and production practices, broiler chickens can weigh 2 kg in about 34 days. Forty-five years ago it would have typically taken over 60 days. These impressive advances have been made using traditional selective breeding methods and more recently by using genomics. Now, with the availability of precision genome engineering tools there are new opportunities to improve poultry production above and beyond those achievable by traditional means. One major opportunity is disease resilience, particularly for viral diseases such as avian influenza that has devastating impacts on the poultry industry. Resilience to specific diseases can be a notoriously difficult trait to select for using traditional breeding and the latest technologies that precisely edit the genome have created new ways to address this challenge.

INTRODUCTION

Precision genome engineering (PGE) tools for rapid and specifically directed change of poultry genomes have created a new approach for the precision breeding of poultry for food production. It is now possible to introduce intra- or inter-species single nucleotide polymorphisms (SNPs) into a chicken line for improved productivity. SNPs and larger

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changes in genetic loci occur spontaneously within individuals in a species and become prevalent or dominant in that species if they confer an advantage through the selective pressures the species is experiencing. With the advent of genetics, molecular biology and genome sequencing it has been possible to apply SNP screening to locate and select for desirable production and disease resistance traits in a range of livestock species. This has enabled a rapid improvement in the development of elite genetics for many species including poultry but in particular the chicken. This still requires a steady and iterative process of breeding, screening and selection. The new era that is ushered in by these new PGE tools means that desirable SNPs, or the gene variants associated with them, can be introduced into a line of genetics in a single step rather than taking several reproductive rounds including screening. This could be the beginning of a new agricultural revolution dramatically reducing the time taken to improve lines of chicken for particular production environments and to introduce resilience to specific diseases that may threaten the security of the food production system. If these SNPs or variants already exist within the species and could be introduced with a longer time frame by conventional breeding, we foresee reduced complexity for regulatory approval of these technologies and their outcomes when compared to more traditional genetic engineering approaches. This should, if communicated clearly and effectively, also challenge the traditional public perception of genetic modification as the new technology delivers precision breeding of intra-species traits compared with the random integration of exogenous genes or traits.

THE CHALLENGE FOR PRECISION GENOME ENGINEERING IN BIRDS

In many animals to date, PGE components for TALEN and CRISPR (DNA, RNA or protein) have been directly injected into the zygote where they target the genome and result in animals carrying edits on one or both chromosomes (1, 2). For improved precision over this process, targeting in somatic cells such as fibroblasts can be used to produce edits, followed by somatic cell nuclear transfer to produce live progeny carrying the mutations (3-5). This has already proven to be very efficient and highly precise and has considerably expanded scientists' ability to make specific alterations to the genomes of a variety of animals.

 http://dx.doi.org/10.2218/natlinstbiosci.1.2016.1742

However, due to differences in reproductive physiology and structure of the fertilised egg and early embryo, many of the relatively straightforward procedures that are used to generate gene-edited fish and mammals are not applicable in avian species. This is because the avian ovum forms with a vitelline membrane which becomes a protective sac around the swelling yolk until it is released and fertilised in the infundibulum at the top of the oviduct. The single-cell zygote is therefore intimately linked with the yolk and subsequently very difficult to manipulate. While recent advances have been made in the areas of avian ova culture and *in vitro* intracytoplasmic sperm injection (6), these techniques are as yet too demanding to consider using for genome editing. Nevertheless, multiple very effective methods have been established for engineering the germline of birds and these are now successfully being developed for gene editing and will lead to rapid advances in the technology in poultry.

METHODS FOR APPLYING GENE EDITING TOOLS IN POULTRY SPECIES

In mammalian species common methods for production of transgenic and gene-edited animals include modification and transplantation of embryonic stem (ES) cells to early blastocysts (7-9), modification of somatic cells in culture for somatic cell nuclear transfer (10-12), or direct delivery of transgenes or gene editing components to *in vitro* fertilised zygotes (13-15). In birds, while early work was done with ES cells (16) the reproductive physiology of avian species greatly restricts access to the ovum or the early blastocyst, thus the methods used in mammals are not used in birds. Generation of transgenic chickens, and later gene-edited chickens has been facilitated by the development of methods to establish primordial germ cell (PGC) cultures. Early in embryogenesis PGCs migrate from the germinal crescent into the bloodstream until they reach the genital ridges, colonise the developing gonads and differentiate into germ cells (reviewed in 17), making them a suitable cell type for genetic modification of the germ line.

Early work with avian PGCs demonstrated that chimeric chickens could be produced by isolating PGCs from donor chickens and directly transferring them into recipient embryos (18) or culturing the PGCs for a short time, then transferring them (19). In 2006 a major breakthrough was made when Van de Lavoir *et al.* (20) demonstrated that chicken PGCs can

be isolated, cultured long term and genetically modified while maintaining their commitment to the germ line. The modified PGCs were transferred to recipient embryos, successfully generating germline chimeras, and breeding of the germline chimeras resulted in transgenic chicks. Since 2006 many different lines of transgenic chickens have been produced through PGC culture (21-24). In addition, a number of techniques which use PGC culture have been employed to generate gene-knockout chickens including homologous recombination (25), TALENS (26) and the CRISPR/Cas 9 system (27,28).

Another recent advancement in PGC technology is the ability to genetically modify PGCs *in vivo* (29). This method involves complexing a plasmid containing a transgene flanked by transposon recognition sites and a plasmid containing the transposase gene with a transfection reagent. The complexed plasmids are then directly injected into the bloodstream of early embryos, transfecting the migrating PGCs on route to the developing gonads. In a portion of the transfected PGCs the transposase will induce transgene integration into the genome. The direct injection method also holds promise for delivery of gene editing components such as TALENs and CRISPR/Cas, however to date no work has been published validating this.

Lack of access to ovum or single celled embryos has in part driven the development of these PGC-based methods. However recent advances have been made in the areas of avian ova recovery, culture and *in vitro* intracytoplasmic sperm injection (6). Researchers were able to recover ova from quail, fertilise them by intracytoplasmic sperm injection, culture the resulting embryos for a day and then transfer the fertilised embryos to surrogate shells, where they lived for up to two additional days. These advances indicate that direct manipulation of avian ovum and single-cell embryos may one day be used for genome editing applications.

Unlike the ovum, avian sperm can easily be collected. However it is difficult to maintain sperm viability once it is collected, making direct genetic manipulation of the sperm genome difficult. Previous research shows that sperm can be efficiently transfected (30) and gene editing tools like TALENs and CRISPR/Cas9 can be used to transfect sperm that can then be delivered during fertilization for editing the single-cell zygote. This process is known as

 http://dx.doi.org/10.2218/natlinstbiosci.1.2016.1742

sperm transfection assisted gene editing, or STAGE, and has been used recently to make gene knockout chickens (31). Being able to use sperm for the generation of gene knockouts opens up this technology to species where PGC culture methods do not exist.

DISEASE RESILIENCE

Disease outbreaks in poultry pose a significant risk to the commercial poultry industry causing devastating loss to the economies of developed and developing countries. Avian Influenza Virus (AIV) is one such destructive and economically important poultry disease due its ability to rapidly re-assort and become hypervirulent causing sporadic pandemic events, often with a high mortality rate (32, 33). Current vaccination strategies using live or inactivated viral vaccine strains to control AIV in poultry is either limited or ineffective as the efficacy is complicated by factors such as age of the bird, health status and antigenic distance of infected virus (34). Therefore, development of feasible and sustainable long-term methods to control emerging pathogens is desirable, and has been a long-standing goal.

Breeding for disease resistance is a very challenging task within the poultry industry. Although genetic variance is one of the major determinants to confer resistance, the practical applications in poultry are yet to be discovered. The rapid progression of genomic resources and next-generation sequencing can allow us to analyse genetic, epigenetic, transcriptomic variations within the species or related species to determine the factors associated with susceptibility and resistance to disease. For instance, the fayoumi chicken is renowned to have resistance against infectious diseases (35). A recent study has identified differential gene expression patterns to AIV infection in two distinct genetic lines of chicken species; the fayoumi and Leghorn chickens. Further investigations on these differentially expressed genes and introduction of those salient genomic variations to the chicken genome using gene editing technology can provide new insights into the production of disease-resistant chickens.

The recent findings of a species-specific host co-factor polymerase activity of avian influenza viruses, the chicken ANP32A (chANP32A) protein, is an intriguing example for understanding disease mechanisms. Although ANP32A is present in humans (huANP32A), the presence of

an additional ~ 33 amino acid insert in chicken ANP32A was shown to be a key contributing factor for the enhanced avian polymerase activity in avian cells (36). Using gene editing technology, substituting the chANP32A gene with huANP32A could impair the enhanced polymerase activity of avian influenza virus in chicken cells, thereby providing resistance to chickens against influenza.

Another clear opportunity to gain insight and potentially identify gene editing targets for disease resilience is via comparative genomics of chickens and ducks. Despite being a closely related species to chicken, the duck possesses very distinctive innate immune responses and resistance to AIV infection (37, 38). The absence of the RIG-I gene in chickens compared with ducks was demonstrated to confer relative susceptibility of chickens to AIV infection (34). The utilisation of genome editing technology in this context, to precisely introduce these RIG-I or RIG-I-like "natural" disease-resistance genes into safe harbor locations in the chicken genome can open the possibility of breeding chickens with increased resistance to influenza.

NEXT-GENERATION OF VACCINE EGGS

Another area in which the genome editing of poultry has the potential for real impact is the production of specialised eggs for vaccine manufacture. Embryonated chicken eggs are used to grow a number of vaccines for both humans and animals. The most common of these is influenza vaccine which has been grown in embryonated chicken eggs since its introduction in the 1940s. Other than advancements in automation and purification, the process to grow influenza in eggs has changed very little over this time and it is known that the growth of the vaccine is limited by the embryo. In some cases up to two eggs can be required for a single dose of influenza vaccine. This means that major vaccine companies can use greater than 1 million eggs per day to meet the requirement for vaccine production.

We now have the ability to not only identify the genes which restrict virus growth using either siRNA or CRISPR whole-genome screens *in vitro*, but we can also edit these genes in the genome of the chicken to produce eggs that could produce higher yields of virus for vaccine production. The ability to produce high vaccine yield eggs would have implications

for the whole vaccine production chain. The ability to reduce the amount of eggs required would reduce transport costs as well as the cost of waste disposal, which can be large as all waste produced from influenza vaccine manufacturing is biological waste that contains live infectious virus.

In addition to reducing the number of eggs needed for influenza vaccine production it may also be possible to improve the eggs in other ways. For some serotypes of influenza their growth in eggs can alter the composition of their haemagglutinin meaning the virus grown in the eggs no longer protects effectively against the wild-type virus. Much like the identification of pro-viral genes, if we are able to identify those responsible for causing the alterations in haemagglutinin we would be able to modify them. These eggs would be capable of growing virus which is more representative of the wild-type virus used to inoculate them and would therefore provide better protection.

Another option is to modify eggs to allow the growth of other viruses which otherwise do not grow well in eggs. Using a whole-genome screen in the same way as described for influenza, genes that inhibit the growth of these viruses could be identified. The chicken genome could then be altered to produce eggs that allow the growth of these virus. This approach could be useful for viruses that are currently difficult to produce vaccines for due to low virus yields. There is also the potential for these eggs which grow other viruses to be processed in the existing infrastructure at influenza vaccine manufacturing plants.

SELECTIVELY HATCHING FEMALE CHICKS

Genetics has contributed to our understanding of the domestication of poultry, likely more than 8,000 thousand years ago (39). It has also contributed to the high performance of the two major type of birds, broilers and layers, used to generate meat and eggs respectively. The dramatic difference in the metabolism of these two types means that male birds generated in the layer industry are no longer commercially viable to grow out for meat in most commercial settings. As a result males are identified following hatch, by manual sexing or feather colour identification, and immediately euthanised with a low value recovery of

http://dx.doi.org/10.2218/natlinstbiosci.1.2016.1742

nutrient from their carcasses. This practice is fraught with ethical issues and incurs costs and production value loss to farmers.

There is a clear need in industry for an alternative that can either identify male chicks before they hatch, preferably at point of lay. The United Egg Producers in the USA have made a statement that they will aim to remove the practice of male culling by 2020 (http://uepcertified.com/united-egg-producers-statement-eliminating-male-chick-culling/). There has been a long history of study into the processes of sexual differentiation during development and into the genes and processes involved (40). While this process is well understood, the key male determining trigger has not yet been proven beyond doubt (41). Other than addition of exogenous hormone (a practice that would not be acceptable to industry or to the consumer) there is little that can be done by way of intervention. Therefore, attention has turned to methods to identify male embryos.

The advent of precision gene editing techniques presents the opportunity to place specific marker genes on the male sex determining chromosome, the Z chromosome. In chicken, and birds in general, the female is the heterogametic sex, carrying one Z and one W chromosome, thus ZW. The male is homogametic, being ZZ, and best evidence indicates that a double dose of the gene DMRT1 on the Z chromosome is key in male development (in the absence of current evidence of a W specific female determining gene). If a marker gene can be site specifically engineered into a safe location on the Z chromosome (Z*) then a breeding pair Z*W (female) crossed with ZZ (male) would yield the following offspring: ZW (f), Z*Z (m), ZZ* (m), ZW (f). So a marker gene on the Z chromosome of a female when crossed to a wild-type male will always yield males carrying the marker gene and females free of the marker gene. This is a null-segregation technique commonly used in plant breeding systems. In a most simplistic set-up the marker gene could be a constitutively expressed green fluorescent protein, such that male embryos even at the point of lay when the embryo is only 60,000 mostly undifferentiated cells, it would be green. Current indications are that with appropriate lasers and detectors this could be detected through the shell of a freshly laid egg without the need for invasive sampling. Since eggs are routinely “candled” to check for viability based on the visualization of venous networks through the shell, a form of light based detection should be able to be adapted to detect the

marker on the males. There are many alternative genes that could be used to provide other means of detection of the mark and the male. The power of this technique is to combine the selectable transgene with the null-segregant exclusion process widely accepted in plant food production (42). This generates wild-type females yielding eggs for the consumer – with the added value of no-“hatch-and-cull” improved production ethics. The farmer also benefits from reduced incubation, egg handling and easier nutrient recovery from males.

INCREASED FOOD SAFETY

Gene editing can be used to improve the food safety of poultry products, namely by removing the allergenic components of chicken eggs. Allergy to chicken egg is a widespread condition affecting up to 2.5% of children and is the second most common food allergy. This presents a major food safety issue for the community since eggs are used in such a wide range of food products. Furthermore, the widespread use of egg-based flu vaccines poses additional risks. The incidence of egg allergy in many parts of the world is increasing and the cause of this is not understood and subject to much debate.

Egg allergy is caused by 4 proteins within the egg white: ovomucoid (Ovm), ovalbumin (Ova), ovotransferrin and lysozyme (43). Ovm is the most allergenic of the four proteins and as such is not surprisingly the first of the allergens to be targeted for gene editing (28). A large amount of research has been carried out to characterise the function of the various egg white proteins, however no clear role has been identified for Ovm in fertility, egg formation or nutritional value. Even though Ovm is the most allergenic egg white protein, it only makes up a small amount of the total egg white protein (~10% compared to Ova which is >50%). The absence of a clear or critical function for Ovm in conjunction with its low abundance may allow for the successful targeted deletion of the Ovm gene in layer hens. Oishi *et al*. (28) have made excellent progress to demonstrate this and have reported the successful generation of a homozygous Omv knockout female chick using CRISPR. We are all now waiting for this bird to reach egg laying age and for a future publication detailing the impact of this gene deletion on Ovm allergenicity, reproductive viability of the hen and physical properties of egg white with respect to processing, cooking and taste.

It will not be possible to delete the genes encoding the remaining 3 allergens (Ova, ovotransferrin and lysozyme). The function of these proteins are well understood and all are critical for the development of an embryo within the egg. The allergenic epitopes within these 3 proteins have been characterised and compared with other avian species including related galliformes and the more distantly related emu (44). Based on this research it may be possible to specifically edit key amino acid sequences within these epitopes to develop hypoallergenic versions of these key genes whilst retaining the critical functionality of each protein. This precise editing is now made possible with PGE tools such as TALEN and CRISPR and opens the possibility of producing allergen-free eggs to eliminate the serious food safety issues associated with egg allergy and to also improve the safety of vaccines that are grown in chicken eggs.

CONCLUSION – IMPACTS FOR THE POULTRY INDUSTRY

The application of PGE in animal agriculture has great potential with many experts predicting that this technology is game-changing with respect to breeding of desired traits in livestock species. It enables the rapid introduction of beneficial, naturally occurring mutations that already exist within a species or closely related species into elite breeding animals. It is precise and does not introduce deleterious or unwanted traits that arise via traditional selective breeding. We now have the technology to create precise, targeted modifications to the chicken genome. The impacts of this can lead to improved efficiency and sustainability of poultry production to help meet the challenges associated with global food security. Specific innovations that result from gene editing technology will lead to new approaches for managing disease, improving welfare, increasing food safety and enhancing the production and safety of vaccines that are grown in chicken eggs. It is possible that the latest developments in gene editing technology may help to reduce or remove the two major barriers to the acceptance and application of genetic engineering technology in animal agriculture: regulatory approval and public perception. This could pave the way for

gene editing and precision breeding to impact on the safe, secure and sustainable production of poultry protein.

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