Reproductive Biotechnology, TUM School of Life Sciences

TECHNISCHE UNIVERSITÄT MÜNCHEN

Doctoral thesis

Generation of androgen receptor knock-out chicken for the investigation of avian sexual development

Kamila Lengyel

TUM School of Life Sciences

Generation of androgen receptor knock-out chicken for the investigation of avian sexual development

Kamila Lengyel

Vollständiger Abdruck der von der
TUM School of Life Sciences
der Technischen Universität München zur Erlangung des akademischen Grades
einer Doktorin der Naturwissenschaften
genehmigten Dissertation.

Vorsitzende/-r: Prof. Dr. Martin Klingenspor

Prüfende/-r der Dissertation:

1. Prof. Dr. Benjamin Schusser

2. Prof. Dr. Manfred Gahr

Die Dissertation wurde am 05.07.2021 bei der Technischen Universität München
eingereicht und durch die TUM School of Life Sciences am 26.10.2021 angenommen.

Reproductive Biotechnology, TUM School of Life Sciences

TECHNISCHE UNIVERSITÄT MÜNCHEN

Doctoral thesis

Generation of androgen receptor knock-out chicken for the investigation of avian sexual development

Generierung von Hühnern mit Androgen-Rezeptor-Knock-Out zur Untersuchung der Geschlechtsentwicklung von Vögeln

Author:	Kamila Lengyel
Supervisor:	Prof. Dr. Benjamin Schusser
Mentor:	Prof. Dr. Manfred Gahr
Submission Date:	01.07.2021

I confirm that this doctoral thesis is my own work and I have documented all sources and material used.

Freising, 01.07.2021 Kamila Lengyel

Acknowledgments

I want to express my gratitude to Prof. Dr. Manfred Gahr and Prof. Dr. Benjamin Schusser for allowing me to work on this project. I want to thank Prof. Dr. Manfred Gahr giving me the position at Max Planck Institute for Ornithology.

Moreover, I want to thank Prof. Dr. Benjamin Schusser for giving me an opportunity to work in his team at Chair of Reproductive Biotechnology. Without his expertise and methodology, this project wouldn't be possible. I want to thank for his great supervision, mentoring, and leadership during the whole project.

I want to thank Prof. Dr. Manfred Gahr for his kind and professional support, scientific insight into this project and being always open to my questions and concerns.

I want to thank all my former and current colleagues Tom Berghof, Denise Duda, Ipek Eroglu, Romina Klinger, Maria Laparidou, Sabrina Schleibinger, Hicham Sid, Theresa Thoma and Hanna Vikkula. I appreciate their help and scientific supports, kind environment, and pleasant time in the office and in the lab.

I want to thank to my colleagues at Max Planck Institute for Ornithology Dr. Albertine Leitão, Dr. Hermina Robotka, Mekhla Rudra and Dr. Lisa Trost for their contribution to this work.

I want to thank to our animal keepers for their work and support in our animal facility in Thallhausen.

I want to thank Nicole Fritz for the proofreading and kind support whenever was need.

I want to thank my whole family for supporting me, for leading and for motivation during my study times.

Last but not least, I want to thank my husband, Jozef Lengyel. Thank you for your unconditional support, patience, and deep understanding. Thank you for always being there for me!

Abstract

Androgens such as testosterone play an essential role in sexual development in most vertebrates. They interact via binding to the androgen receptor (AR), which leads to a direct change in gene expression. Mutations of androgen receptor causing a knock-out are well described in humans and mice. Furthermore, in mice, several knock-out models of the androgen receptor were established, showing the importance of androgens not just in sexual development but as well as in the immune system, metabolism, and behavior. In birds, no androgen receptor knock-out model has been generated yet. In this project chicken, primordial germ cells (PGCs) have been modified using the CRISPR/Cas9 system and homologous directed repair in order to knock-out the chicken androgen receptor.

Here the question arises how the lack of functional AR/androgen signaling influences the sexual development of chickens, which phenotype can be expected, and how the male and female androgen receptor knock-out birds will be affected.

Gene-edited primordial germ cells were injected into chicken embryos to generate germline chimera. Subsequently, androgen receptor knock-out (ARKO) chickens have been generated by the breeding of germline chimeras. In addition to androgen receptor knock-out, strategies for the generation of AR reporter chicken line were developed. By fusion of AR with a tag or fluorescent protein, the AR expression can be localized in particular tissues and in different stages of chicken development. Our experiment proved that tagging of AR with flag tag or mCherry does not interfere with the AR signaling.

ARKO$^{-/-}$ male and female chickens exhibited a complete lack of sex dimorphisms and showed complete infertility. Furthermore, we have demonstrated the nonfunctionally of the androgen receptor/androgen signaling by dipping chicken eggs of knock-out and WT birds in testosterone solution and examined the development of the bursa of Fabricius. In treated WT embryos, bursae showed rapid involution, while the bursae of ARKO$^{-/-}$ chicken remained unchanged. Moreover, the composition of peripheral blood mononuclear cells (PBMC) in ARKO$^{-/-}$ and WT chickens was measured. ARKO$^{-/-}$ roosters did not show any difference compared to wild type birds. In ARKO$^{-/-}$ hens a significant decrease was found in all measured lymphocyte populations. The ARKO chicken line gives an interesting insight into the chicken sexual development, which can be further investigated in following experiments.

Kurzfassung

Androgene (Testosteron) spielen eine bedeutende Rolle in der Geschlechtsentwicklung der meisten Wirbeltiere. Sie interagieren über die Bindung an den Androgen-Rezeptor (AR), was zu einer direkten Veränderung der Genexpression führt. Mutationen des Androgen-Rezeptors, die zu einem Knock-out führen, sind im Menschen und in Mäusen gut beschrieben. Darüber hinaus wurden in der Maus mehrere Knock-out-Modelle des Androgen-Rezeptors etabliert, die die Bedeutung von Androgenen nicht nur für die Geschlechtsentwicklung, sondern auch für das Immunsystem, den Stoffwechsel und das Verhalten zeigen. Bei Vögeln wurde bisher kein Androgen-Rezeptor-Knock-out (ARKO) Modell generiert. In diesem Projekt wurden primordiale Keimzellen (PGCs) des Huhns mithilfe des CRISPR/Cas9-Systems in Kombination mit homologer Rekombination modifiziert, um den Androgen-Rezeptor im Huhn auszuschalten.

Hier stellt sich die Frage, wie sich das Fehlen von funktionierendem Androgenrezeptor/ Androgen-Signaling auf die sexuelle Entwicklung von Hühnern auswirkt, welcher Phenotyp erwartet werden kann und wie die männlichen und weiblichen Vögel mit beeinträchtigtem Androgenrezeptor (ARKO) betroffen sind. Abgesehen von sekundären Geschlechtsmerkmalen, welches sexuelle Verhalten können wir bei männlichen und weiblichen ARKO erwarten? Wie beeinflusst das dysfunktionale Androgen-Milieu das Immunsystem von Hühnern?

Genetisch veränderte primordiale Keimzellen wurden in Hühnerembryonen injiziert, um Keimbahnchimären zu erzeugen. Anschließend wurden Androgen-Rezeptor-Knock-out Hühner durch Verpaarung der Keimbahnchimären erzeugt. Zusätzlich zum Androgen-Rezeptor-Knock-out wurden Strategien zur Erstellung einer Androgen-Rezeptor-Reporter-Hühnerlinie angewandt. Durch die Fusion des AR mit einem Tag oder einem fluoreszierenden Protein kann die AR-Expression in bestimmten Geweben und in unterschiedlichen Stadien der Hühnerentwicklung lokalisiert werden. Unser Experiment zeigte, dass die Markierung des ARs mit einem FLAG-Tag oder mit mCherry das AR-Signaling nicht beeinträchtigt.

Männliche und weibliche ARKO$^{-/-}$ Hühner wiesen ein komplettes Fehlen des Geschlechtsdimorphismus auf und waren Unfruchtbar. Darüber hinaus haben wir die fehlende Funktion des AR bzw. der Androgen-Signalweiterleitung nachgewiesen, indem Hühnereier der Knock-out und WT Vögel in Testosteron-Lösung getaucht und die Entwicklung der Bursa Fabricius untersucht wurde. In den behandelten WT-Embryonen

zeigte die Bursa eine schnelle Involution, während die Bursa der ARKO$^{-/-}$-Hühner unverändert blieben. Außerdem wurde die Zusammensetzung der PBMCs in ARKO$^{-/-}$- und WT-Hühnern gemessen. ARKO$^{-/-}$-Hähne zeigten keinen Unterschied im Vergleich zu den WT-Vögeln auf. Bei ARKO$^{-/-}$-Hennen wurde eine signifikante Abnahme alle gemessener Lymphozytenpopulationen festgestellt. Die ARKO-Hühnerlinie ermöglichte einen interessanten Einblick in die Geschlechtsentwicklung des Huhns, welche in folgenden Experimenten weiter untersucht werden kann.

Contents

1. Introduction

Sex development and sex determination were always in the focus of biomedical research and agriculture. As there are so many different kinds of organisms, it is not surprising that their sex determination mechanisms differ a lot. While for all crocodilians and some amphibians the sex is determined by the incubation temperature of an egg, in most vertebrates, including mammals, the sex is dependent on chromosomal inheritance. Mammalian sex is determined by gene SRY located on the Y chromosome. Its expression is essential for testes differentiation and further biochemical processes leading to androgen production [1]. Therefore, it is not surprising that androgens are one of the key players in male sexual maturation and dimorphism. Their actions are mediated by binding to the androgen receptor (AR) [2]. Particularly interesting is that a dysfunctional androgen/AR system causes female-like phenotype in male mammals, lack of secondary dimorphism, or infertility. Such a condition is known as androgen insensitivity syndrome or syndrome of testicular feminization [3, 4]. Thus, in mammals, genomic sex determines the gonadal sex which then, through hormonal secretion, affects all somatic tissues that express androgen receptors [5].

With the development of molecular biology techniques, it has been discovered that androgen insensitivity syndrome is caused by mutations in androgen receptor [6]. This has opened up a new era in generating androgen receptor knock-out models in mice, with emphasis on how androgens affect male and female development [7, 8, 9, 10]. From these studies, it became evident that androgen/AR signaling affects reproduction, metabolism, immunity, and behavior in males and female mammals [11, 12, 13, 14].

In mammals, it is well accepted that hormonal secretions from the gonads directly influences the phenotype, in avian species cells possess so-called autonomous sexual identity and might developed independently from sex determining factors [15]. In particular, due to the lack of Z-chromosome inactivation in male birds throughout all tissues (in a difference to the X-chromosome inactivation of female mammals), sex-specific expression of genes located on the sex chromosomes would affect all body tissues. Thus, in birds, somatic sex is possible without hormonal secretion of the gonads such as androgens [15]. Based on these findings, it appears that chicken sexual development of somatic tissues is a complex process, influenced by genetic cell-autonomous mechanisms as well as by hormones [5, 16]. Thus, sexual development might be possible without androgen signaling.

In this thesis, two main research topics are presented: 1) the development of an androgen receptor knock-out (ARKO) chicken to analyze the role of androgens in avian sexual development, and 2) the generation of an androgen receptor reporter chicken line. One of the essential questions asked in the experiments below was, whether a similar androgen-sensitive phenotype in male chicken could be observed as in ARKO male mammals. Further, I compare male and female ARKO chickens to verify, whether the ARKO will have the same impact, e.g., on their fertility and sexual dimorphism.

2. Literature

2.1. Endocrine regulation of male and female reproduction

This chapter briefly summarizes the endocrine regulation of sex development in humans and mice, describes spermatogenesis and oogenesis, and illustrates the importance of androgens during reproduction and as well its influence on other body systems.

2.1.1. Hypothalamic-pituitary-adrenal axis

Endocrine regulation is essential in male and female reproduction. The key player, the hypothalamus, is releasing gonadotropin-releasing hormone (GnRH). GnRH stimulates the pituitary gland to synthesize the luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH does not directly release the sex steroid hormones like androgens, estrogens, or gestagens but instead stimulates the pituitary gland in the excretion of LH. In both sexes, LH and FSH stimulate gametogenesis and promote the synthesis of sexual steroids. In females LH, together with FSH, stimulates the estrogen synthesis. In addition, LH is responsible for the rupture of the follicle wall and ovulation. In males, LH affects Leydig cells to release the primary testicular hormone, testosterone [17, 18]. Scheme of the hypothalamic-pituitary regulation is displayed in the Figure 2.1.

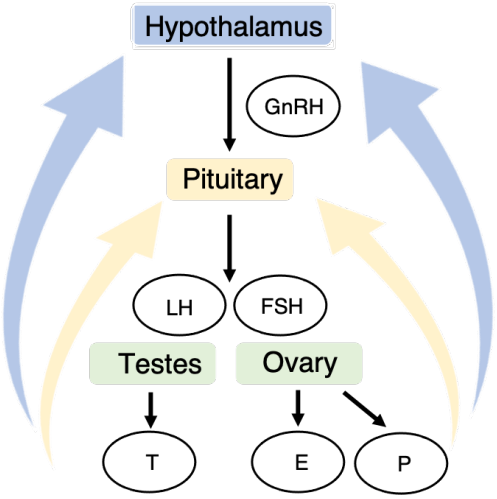

Figure 2.1.: **Hypothalamic-pituitary system**, Hypothalamus releasing GnRH acts on the pituitary to synthesize LH and FSH. LH and FSH stimulate the reproductive organs in the production of sex steroids. Production of testosterone and estrogen gives negative feedback and decrease the synthesis of LH, FSH, and GnRH, Testosterone (T) , estrogen (E) , progesterone (P) , modified from [17].

Estrogens, estradiol, and the metabolically active form estrone and estriol promote sexual behavior and are responsible for secondary sex characteristics. Testosterone is produced mainly by Leydig cells with limited production in the cortex of the adrenal glands. After entering the cell, testosterone is converted into dihydrotestosterone (DHT), binding to the AR. As in females, sex steroids in males are responsible for secondary sex characteristics, sexual behavior, and gametogenesis [17, 18].

Regulation of the hypothalamic-pituitary system is based on so-called negative or inhibitory feedback. Sex steroids act to decrease the levels of LH and FSH, which give a signal to the hypothalamus to decline GnRH production. Estrogen and progesterone act on the hypothalamic-pituitary system with regards to the phase of the menstruation cycle [17, 18].

2.1.2. Spermatogenesis and oogenesis

On a macroscopic level, testes are divided into sections (lobules) separated by connective tissue. Each lobule is organized in seminiferous tubules surrounded by a basement membrane underlined by myoid and Sertoli cells. The role of Sertoli cells is to nourish and support germ cell development [19, 20]. Sertoli cells are also the blood-testis barrier

protecting the germ cell antigens to be recognized by the immune system [17]. Leydig cells (producing testosterone) and blood vessels are found in the interstitium [19].

Spermatogenesis (Figure 2.2) is a process of cell division and differentiation of spermatogonial cells into spermatocytes. Diploid spermatogonia A firstly go through several mitotic division to give rise to spermatogonia B and primary spermatocyte. Primary spermatocytes then enter meiosis I to form a secondary spermatocyte. The last part of the spermatogenesis is called spermiogenesis since secondary spermatocytes during the meiosis II differentiate in spermatozoa and mature sperms. During the whole process, developing germ cells migrate from the basement membrane to the luminal side of the seminiferous tubules, where the sperms are released into epididymis [17, 19, 20].

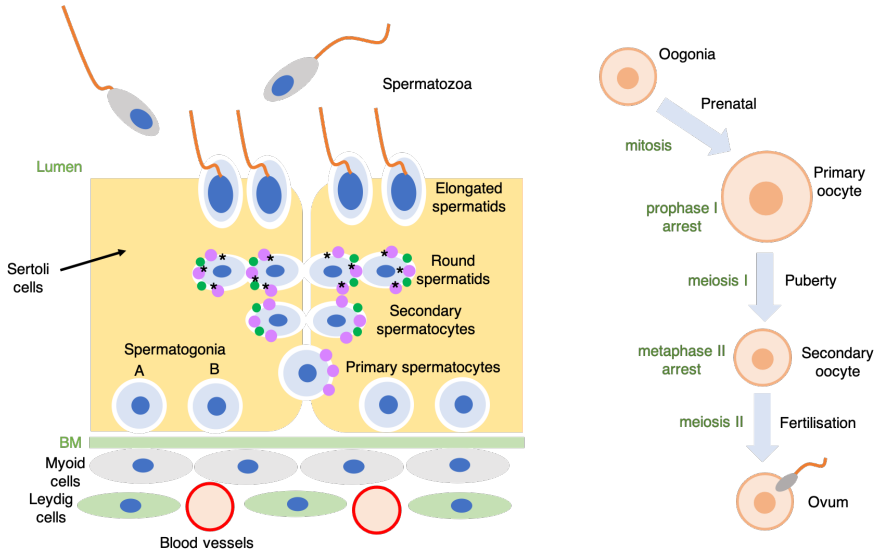

Figure 2.2.: **Spermatogenesis and oogenesis**, Spermatogenesis (left side) is a process localized in seminiferous tubules. Spermatogonia A and B enter meiosis to form primary and secondary spermatocytes. The final meiotic division then gives rise to spermatids and spermatozoa, which are released through the lumen of the seminiferous tubules into the epididymis. Oogenesis (right side) starts already in prenatal development, where oogonia differentiate in the primary oocyte. Oogenesis continues then in puberty, and during ovulation, the secondary oocyte is released. In case of fertilization maturation of the oocyte is completed, modified from [19, 21].

The structure of the ovaries consists of three parts, the peripheral part, the cortex, where the folliculogenesis takes place, and the central region, medulla, filled with connective tissue, vessels, and nerves [17].

Oogenesis (Figure 2.2) is the process of differentiation of the germ cells into a matured egg cell. At first primordial germ cells divide via mitosis to form oogonia. Oogonia then enter meiosis I and form the primary oocyte. Its development is arrested in the prophase of meiosis I. Differentiation of the primary oocytes is restricted to embryonic development, and continues further in puberty. From about seven million of prenatal oogonia, just two survive until birth, with a further decline to 300 000 per ovary on the onset of puberty [17, 21]. Meiosis I is completed during the ovulation forming secondary oocyte, which enters meiosis II and goes through meiotic arrest (metaphase). Just in case of fertilization, the complete, mature oocyte is developed [17, 18, 21].

2.1.3. The role of androgens in mouse and human physiology

Over the years, it became evident that androgens are essential in male and female reproductive physiology. Nevertheless, it is necessary to underline that androgens are involved in other processes. This chapter summarizes the influence of impaired AR/androgen signaling on metabolism and the immune and cardiovascular systems.

2.1.3.1. Androgens in metabolism of glucose and fat

It is well known that testosterone levels correlate with abdominal obesity in men as a risk factor for cardiovascular diseases or metabolic syndrome. Several studies on androgen receptor knock-out (ARKO) mice showed that lack of the androgen receptor causes metabolic imbalances. Lin *et al.* reported increasing glucose and insulin levels from the 20^{th} week of age compared to the wildtype (WT) mice leading to insulin resistance. Furthermore, increased triglyceride deposition and leptin levels were in a linear relationship with increasing body weight. After administering exogenous leptin with the same feed intake, the WT's body weight has significantly decreased compared to the ARKO mouse. Furthermore, ARKO mice exhibit a higher fat percentage with an accumulation of visceral fat [11, 12].

2.1.3.2. Influence of androgens on immune system in androgen receptor knock-out mice models

Several studies reported a potential influx of AR/androgens on innate and adaptive immunity [13]. Chuang *et al.* observed that global ARKO mice developed neutropenia and exhibited almost 90% reduction of neutrophil counts compared to the wild type mice. Furthermore, in this study, the neutrophil population was measured in neutered mice and mice with the syndrome of testicular feminization (*tfm*). In *tfm* mice and neutered mice, similar results were found, although neutered mice were able to restore

the neutrophil population after administration of androgens. In the challenge experiment, the ARKO mice were found more susceptible to bacterial infection than the wild type mice, indicating that the reduced neutrophil population had a severe effect on innate immune response [22]. The population of the monocytes was also affected by AR/androgens actions during the inflammatory response. Monocytes infiltrating into the damaged tissue differentiate into macrophages to do phagocytosis and produce pro-inflammatory cytokines. Interestingly, neutering of animals or blocking the androgen action resulted in accelerated wound healing and suppressed recruitment of macrophages in the damaged tissue [23].

B-cells and T-cells play a significant role in the adaptive immune response. The thymus is the major organ for T-cells development. It was observed before that with puberty and the production of sexual hormones, the T-cell population is significantly decreased. Ashcroft *et al.* showed that neutering of mice caused thymic enlargement, which was reversed by administration of androgens. Furthermore, thymic enlargement was observed as well in ARKO mice as well as by mice with natural AR disorder [23]. Effects of AR/androgen on B-cells were also observed. Altuwaijri *et al.* reported that neutering or impaired function of AR (ARKO) in mice caused spleen enlargement and an expanded B-cell population due to the higher ability of B-cell to resist apoptosis. A ubiquitous AR knockout led to increased B-cell lymphopoiesis, indicating that androgen/AR signaling might play an important role in regulating lymphopoiesis. Nevertheless, in mice with B-cell specific knock-out of AR, only a mild effect on lymphopoiesis was observed [13, 24].

2.1.3.3. Impaired AR/androgens signaling and its effect on behavior

The testosterone actions are essential for masculinization of the male brain in prenatal and postnatal development leading to male-typical behavior in adulthood. Sato *et al.* examined sexual behavior in ARKO mice and revelated those males did not exhibit any sexual behavior. However, it was not completely clear if this is a cause of failed masculinization in the perinatal stage or impaired function of AR/androgen signaling in adulthood. To address this question, WT female mice were treated with dihydrotestosterone (DHT) in a perinatal stage. DHT treatment-induced brain masculinization and male-typical behavior in adulthood, while in ARKO female null mutant (homozygous) mice, brain masculinization was abolished. These findings suggested that androgen/AR action during the prenatal and perinatal stage are more important for further male behavior development [14].

2.1.3.4. Impaired androgen signaling affect the cardiovascular system

The effect of the nonfunctional androgen/AR system was studied in the cardiovascular system since AR expression was discovered in mammalian cardiomyocytes. For example, in neutered animals, suppressed cardiac hypertrophy and fibrosis (one of the significant risk factor for cardiac failure) was observed [25]. After the establishment of ARKO models, these patterns were studied on a deeper level. Ikeda *et al.* examined influx of ARKO on the cardiovascular system with exogenous administration of angiotensin II (Ang II) as a tool for inducing cardiac fibrosis, hypertension, and heart failure. Besides other findings, the heart weight of ARKO mice was significantly smaller than in WTs. Administration of Ang II (hypertrophy induction) caused an increase in weight of both (WT and ARKO), nevertheless the heart weight of heart at ARKOs, remained smaller than at WT mice. Moreover, histological analysis revealed that ARKO mice Ang II cause atypical development of cardiac hypertrophy, indicating that the androgen/AR system is essential for physiological hypertrophy development [26].

2.2. The androgen/androgen receptor signaling and their role in reproduction

In humans or mice, testosterone belongs to the primary excreting androgen. The majority of testosterone production is located in Leydig cells in the testes, but partial production can also be found in adrenal glands. Other steroids as androsterone or androstenol are produced in sweat glands and do not possess biological activity [2].

Testosterone or its metabolite, dihydrotestosterone, acts via binding to the androgen receptor (AR) [2]. AR receptor consists of eight exon coding three structurally and functionally diverse domains: NH2 – terminal domain (NTD), DNA-binding domain (DBD), ligand-binding domain (LBD), and hinge region situated between DBD and LBD [27]. AR is located on the X chromosome in humans and mice; thus, a mutation in males results directly in an androgen insensitivity phenotype (described in more detail in chapter 2.2.2) since only one copy is present [28, 29]. Mutations of AR beside androgen insensitivity syndrome result in Kennedy's disease caused by an increased number of CAG repeats in exon 1 [30]. Kennedy's disease is demonstrated by spinal and muscular atrophy, while at the same time, patients develop symptoms of androgen insensitivity [30, 31].

Androgen receptor is widely expressed in almost all tissues beside spleen. And its transcription is a mediated by variety of transcriptional activator [30]. In chicken, the cDNA sequence of androgen receptor was firstly identified by Katoh *et al.* The most conserved among all species is DNA-binding domain, while ligand binding domain

shares between 60 and 92% of the sequence. The least conserved domain is N-terminal domain having only maximum 30% of homology. Moreover, the expression in different tissues was in chicken embryos analyzed. Surprisingly, significantly higher expression was found in female gonads and syrinx at embryonic day 15 than in males [28]. The structure of chicken androgen receptor is displays in the Figure 2.3.

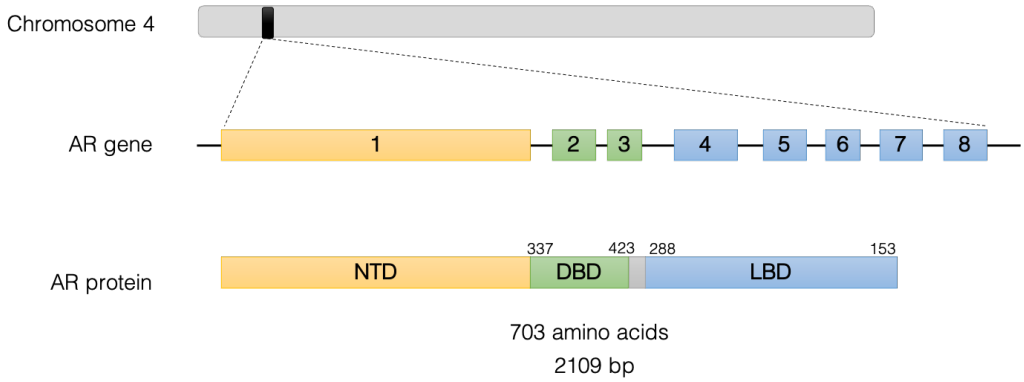

Figure 2.3.: **Location and gene structure AR** In chicken, AR is located on chromosome 4 and consist of 3 structural domains, NH2-terminal domain (NTD), DNA binding domain (DNA), ligand-binding domain (LBD), modified from [28]

2.2.1. Androgen receptor signaling

Androgen receptor (AR) is located in the cytoplasm in a complex with three heat shock proteins (HSP) and other chaperon proteins [32, 33]. Once testosterone (T) crosses the cytoplasmatic membrane, it is converted to dihydrotestosterone (DTH), exhibiting higher affinity (approximately 2 to 10-fold) to the AR than T. Therefore, naturally, much lower concentrations of DTH are necessary for activation of AR. The ligand binding to the AR is followed by conformational changes and dissociation of the AR from the chaperon complex. Activated androgen/AR complex translocates to the nucleus. In the nucleus, the androgen/AR complex binds to the specific recognition sequences called androgen response elements (AREs) in the promoter and enhancer regions of target genes resulting in modulation of gene expression [32, 33, 34]. Figure 2.4 displays the signaling process.

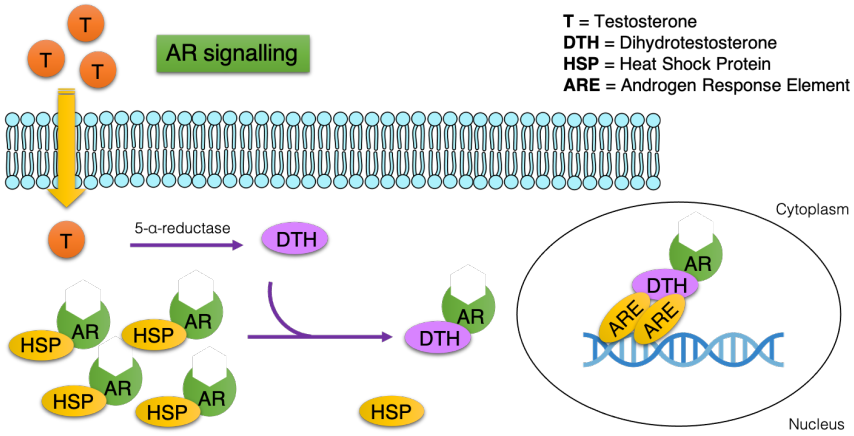

Figure 2.4.: **Scheme of the AR signaling**, AR is localized in the cytoplasm bound to the heat shock protein. After testosterone (T) the cell, it is converted to dihydrotestosterone (DTH). DHT activates the AR, and the whole ligand-receptor complex translocates to the nucleus while binding to the androgen response elements (AREs) on target genes, modified from [32].

2.2.2. Reproduction in androgen receptor knock-out models

Naturally occurring defects of AR were reported during the 1800's and early 1900's [3]. Manifestation with reduced or absent effects of androgens has been later called as syndrome of testicular feminization (*tfm*) or androgen insensitivity syndrome (AIS) [3, 4]. AIS carries a broad range of clinical features from complete androgen insensitivity syndrome (CAIS) to partial androgen insensitivity syndrome (PAIS) depending on the activity or inactivity of AR. Males with *tfm* were strongly affected. They typically expressed female somatic and external genital morphology. Furthermore, internal male and female genitalia like epididymis, prostate or ovary, and uterus were completely absent [4, 35]. Although the fetal Leydig cell population developed regularly (hormone-independent), a function of adult Leydig cell complement was strongly impaired, and its number was reduced [36]. Spermatogenesis was as well affected with spermatocytes arrested in the pachytene stage of meiosis I, and blood levels of testosterone were reduced. In *tfm* females the AR impaired function manifests as XO genotype with direct mutation of AR. This very rare phenotype exhibited a reduced number of follicles with subfertility. Nevertheless, AR/androgen-mediated imbalance was not essential in ovulation, pregnancy, or lactation [4, 35, 37].

2.2.3. Global androgen receptor knock-out mice models

The suitable female *tfm*/ ARKO model was over the years challenging and very difficult to describe since female knock-outs could not be generated by natural mating because of male infertility [4]. Therefore Cre/loxP system was used to create ARKO conditional knock-outs. Cre recombinase is an enzyme allowing to excise DNA located between specific sequences called loxP sites. The exon of AR chosen for the knock-out was surrounded with loxP sites (´floxed´), but at the same time its function remained unchanged. Then, Cre-expressing mice were bred to mice carrying AR-loxP to generate ARKO [4, 38].

Several global knock-out mice models of AR were generated with different Cre-recombinase promoters. The knock-out of exon 1 [7, 8] or exon 2 [9, 39] with insertion of premature stop codon leading to extensive loss of AR function was most efficient. By the in-frame deletion [10] of only exon 3, truncated but non-functional AR protein was found. In general, all global knock-out mice models of AR phenotypically exhibit similar phenotype as seen by spontaneous CAIS or *tfm* [4].

In male ARKO models numbers of Sertoli and Leydig cells were reduced. Levels of testosterone in blood were significantly decreased (as observed in *tfm*) with reduced expression of 17αhydroxylase essential for testosterone synthesis. As for *tfm* , global ARKO mice had small intra-abdominally positioned testes with a lack of secondary reproductive organs. All males were infertile with spermatogenesis arrest at meiosis I pachytene stage [4, 38].

Female ARKO mice appeared to be less affected by lack of AR/androgen function depending on, which exon was knocked-out in certain mouse model and how much the signaling was altered. Females exhibited normal development of ovaries, oviduct, and uterus, although some models expressed reduced uterus diameter [40, 41]. Follicle's development was defective, with dysfunctional ovulation rates causing subfertility. Although AR/androgen action seems essential for uterine reproductive function, implantation and fetal development were not affected by lack of AR function. Surprisingly, knock-out of exon 3 of AR did not affect the growing follicles population and ovulation rates, although the hypothalamic-pituitary regulation was impaired with defect of negative feedback signaling (increased level of FSH and E2) [4].

2.2.3.1. Cell-specific androgen receptor knock-out mice models

Several scientific groups have developed mice carrying ARKO but in specific cell population [8, 39, 42, 43]. These animals were generated via Cre/loxP system. Cre expression was driven by tissue or cell-specific promoter, while in other tissues or cell types, the function of AR should remain unchanged.

Knock-out of AR in Sertoli cells (SCARKO) was generated in several studies [8, 39, 44, 45]. Sertoli cells nourish the germ cells and progress the spermatogenesis process while expressing FSH receptor and AR. In few studies, Cre expression was driven by Anti-Müllerian hormone (AMH) promoter expressed exclusively in Sertoli cells [8, 39, 45]. Lim *et al.* have used ABP (androgen-binding protein) promoter [44] to knock-out of exon 1, 2 or 3 of androgen receptor gene. SCARKO models, showed normal external male appearance with developed male genitalia. The testes were fully descendent, but their size was decreased. Spermatogenesis was blocked in meiosis I (pachytene or diplotene stage) with no presence of sperms in the epididymis. Testosterone levels were normal or slightly reduced depending on knock-out exon or used promoter. Interestingly compared to global ARKO, in SCARKO, normal Sertoli cell populations were observed, suggesting that AR is not necessary for their development [4, 46].

Leydig cells are primarily determined for the production and secretion of testosterone. **Leydig cells knock-out** (LARKO) were generated by breeding with Cre expressing animals under the Anti-Müllerian hormone receptor-2 (Amhr2), which is expressed in Leydig cells, but it was also found in the seminiferous tubules [43]. LARKO mice expressed a smaller size of testes and epididymis in comparison to WT animals. Furthermore, spermatogenesis was as well affected with spermatocytes in pachytene (meiosis I). Levels of testosterone in blood were significantly decreased, while LH and FSH were increased [4, 38, 46]. Surprisingly, Xu *et al.* reported at LARKO normal mating behavior with copulatory plug formation, despite deficient testosterone levels [42].

Selective knock-out in germ cells was (GARKO) conducted by Tsai *et al.* In this study, exon 2 was 'floxed,' and knock-out mice were generated by breeding with animals expressing Cre driven by Synaptonemal Complex Protein 1 promoter a typical germ cell marker. GARKO mice showed normal fertility with normal blood levels of testosterone [47].

Overall taken, selective cell knock-out models (besides GARKO) were infertile with reduced testes size. Many other specific cell ARKOs were generated. As a representative example, Sertoli cell, Leydig cell, and germ cell knock-out were described [4, 38, 46]. In females, no specific cell knock-out was generated until now. Faith of AR in tissues like the uterus or mammary gland in females can be studied via Cre expressing tissue-specific promoters [4].

2.3. Sexual dimorphism and sex determination in chicken

The sex of birds and mammals is determined by chromosomal inheritance. In mammals, females are homogametic sex characterized by XX, while males are heterogametic sex

carrying XY. When eggs are fertilized by sperm carrying X or Y chromosome, female respectively, a male phenotype is developed. The sex-determining factor has been identified already more than 30 years ago. The gene SRY was identified as a testis-determining factor, and it is essential for testis development in XY embryos. SRY is expressed initially in embryonic gonad inducing the hormone production and followed by sex development in other organ systems [48]. Male sex determinant was also confirmed by Koopman *et al.*. In this study, testis development was observed at female genetic mice (XX) after introducing human Sry in mice genome [49]. When the SRY is not present, specific cascades lead the development towards female phenotype [50].

In contrast, in birds females (heterogametic sex) are characterized by WZ and males (homogametic sex) by ZZ chromosomes, but mechanism of sex determination and sex development is still unknown. It is necessary to stretch out that the Z chromosome is not inactivated in any tissue of birds as, for instance, X chromosome in mammalian females is. Complete lack of Z inactivation potentially leads to sex differences without the need for hormone signaling of the gonads [15, 51].

This chapter describes briefly gonad development in chicken and possible candidates for sex determination.

2.3.1. Gonad development in chicken

In chicken, embryonic development takes 21 days. In 1951 chicken embryonic development was staged by Hamburger and Hamilton (H&H) resulting in 45 developmental stages. Gonad development in chicken is widely conserved and similar to other vertebrates. Brief scheme of gonad development is displayed in the Figure 2.5. On day 3.5 (H&H stage 20), gonad consists of the outer epithelium - cortex and underlying medulla. At this point, primordial germ cells (PGCs-precursors for sperms and eggs) migrate through the bloodstream and colonize the gonads, but gonads are still morphological identical [52].

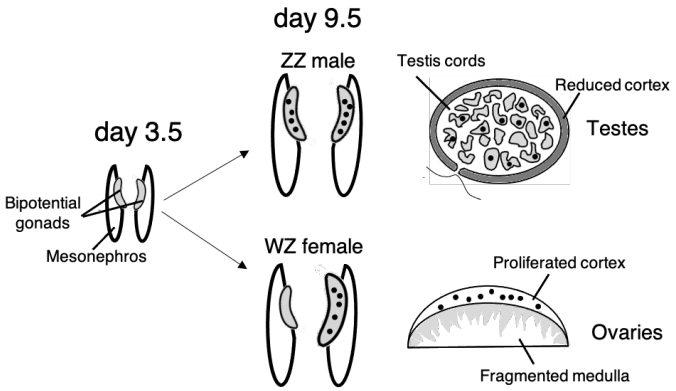

Figure 2.5.: **Development of the gonad during embryogenesis**, At the beginning of chicken embryonic development (3.5 days), chicken gonads are still indifferent and indistinguishable. With an expression of several genes and release of hormones, male and female gonads further differentiate. The female right gonad completely regresses, while the left gonad develops in the functional ovary. Males develop bipolar gonads, which both grow into testes, modified from [53].

In between embryonic days 4.5 to 6.5 (H&H stage 25-30), cortex and medulla proliferate further, and gonads become distinct on the histological level. Male (ZZ) embryos differentiate in bilateral testes. The interior part (medulla), while thickening, develops in seminiferous tubules with Sertoli cells, supporting, protecting, and nourishing germ cells. In contrast, the exterior gonad part (cortex) is reduced. Pre-Sertoli cells produce an Anti-Müllerian hormone (AMH) and cause regression of embryonic oviduct (Müllerian duct). In male embryos, germ cells are primarily localized in the medulla and differentiate in pro-spermatogonia. Then the mitotic arrest occurs, and meiosis continues after hatch [53, 54].

As mentioned at 6.5 days of development, female gonads become morphologically different from male gonads. Female embryos (ZW) show asymmetric gonad development. The right gonad regress, while the left gonad develops into ovaries. In contrast to male embryos, the cortex in females is thickened while the medulla becomes fragmented. Germ cells accumulate strongly in the cortex and enter the meiotic prophase starting the folliculogenesis. Between 6.5 and 7 days of development, the aromatase expression is initiated, occurring just in female embryos and turning androgens to 17-β-estradiol [52, 53].

2.3.2. Genes involved in chicken

Although the sex determination in birds was extensively studied, the molecular mechanism of sex determination in birds remains unknown. Two general hypotheses were established describing possible sex determination. Firstly, the Z dosage hypothesis suggesting that sex is determined by the dosage expression of one or more genes present on the Z chromosome. Secondly, the hypothesis of dominant sex determining genes suggests that on the female W chromosome an ovary determining factor is located. These two hypotheses are not necessarily mutually exclusive [48, 54].

In males, one of the strongest candidates for sex determination is Doublesex and mab-3-related transcription factor 1 (DMRT1). DMRT1 is localized on the Z chromosome with no homolog on female chromosome W. The expression is firstly observed at embryonic day 3.5 in embryonic gonads (specifically in the medulla) [53, 54]. Knockdown of the DMRT1 in early embryos with RNA interference (RNAi) led to feminization and partial sex reversal of the genetically male (ZZ) embryos [55]. Ioannidis *et al.* reported knock-out of DMRT1 on one allele, while the other one provided functional protein. Heterozygous roosters developed testes on right sight and ovaries on the left side. These publications showed that DMRT1 is necessary to be expressed in double dosage for developing functions testes [16]. Expression of DMRT1 is followed by activation of Sex determining region Y-box 9 (SOX9), leading to testes development. SOX9 is expressed from day six only and regulates Sertoli cells differentiation and seminiferous chords formation [55]. Expression of SOX9 precedes the expression of AMH (Anti-Müllerian Hormone). AMH is widely conserved in vertebrates, also primarily linked to Müllerian duct regression, which otherwise develops in ovaries. AMH is also expressed in the right female gonad, which regresses [53, 54]. Scheme of the gene expression at different stages of development is display in the Figure 2.6.

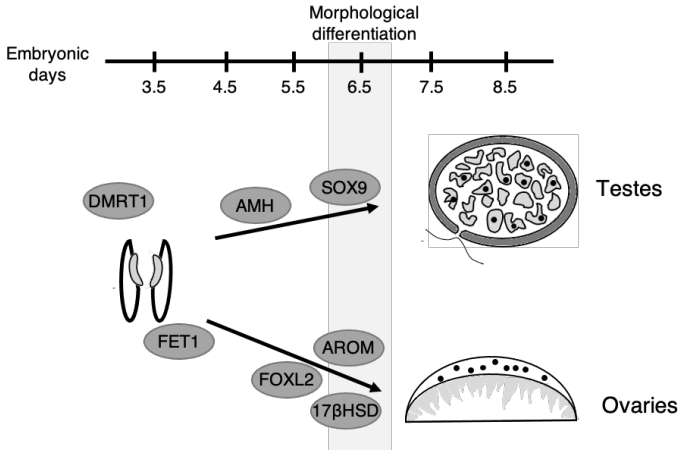

Figure 2.6.: **Genes involved in sex differentiation**, In chicken, primary sex determinant is still not discovered. One of the strong candidates is DMRT1, which expression was found in males around 3.5 days old embryo in gonads. DMRT1 then promotes the expression AMH or SOX9, supporting the testes development. In females, no direct ovarian determinant was found. Fundamental is expression of aromatase and 17 β-hydroxysteroid dehydrogenase producing female sex steroids, modified from [53].

In females, Female-expressed transcript (FET1) is expressed from embryonic day 3.5-4.5 in the left developing ovary. This gene is W-linked and does not appear to have a homolog on the Z chromosome, and no orthologs were found in other bird species. A key player in further female gonad development plays Forkhead Box L2 (FOXL2). FOXL2 is expressed in gonads from embryonic day 5 (stage 28), but since its location is autosomal, it can't be considered as a primary sex-determining factor. Expression of FOXL2 is highly associated with aromatase (CYP19A1) expression during the whole ovary development from embryonic day 4.7-12.7, a suggestion that FOXL2 might be an aromatase regulator. Aromatase is required for ovarian development since together with 17 β-hydroxysteroid dehydrogenase (17βHSD) convert androgen to estradiol [53, 54].

2.4. Effect of androgens on avian physiology and behaviour

2.4.1. Androgens in body composition and syrinx development

Several studies have examined the effect of caponization (castration) or testosterone administration in chickens in order to decrease or increase the level of testosterone and compare the results with control animals. The following text summarizes some of

the evidence from case studies with the effect of androgens on body composition, fat metabolism, and syrinx development.

In general, androgen increases the protein synthesis while at the same time reduces the amino acid catabolism, thereby increasing muscle mass [56]. So, one could expect a similar anabolic effect also in birds. In contrast to this hypothesis, Fennell and Scanes examined the effect of androgens on muscle growth, but no anabolic effect was found in chickens [57]. *In ovo* testosterone administration did not seem to affect the weight gain or muscle development in chicken after hatch [58].

Yet, it is well documented that caponization in chicken or pheasant can change fat metabolism. Caponization is an old technique used to improve meat quality. The removal of the testes increases fat content in the abdominal, subcutaneous, and intramuscular areas [59, 60, 61]. Furthermore, it was reported that capons (castrated roosters) had heavier liver, higher cholesterol, and high density lipoproteins serum levels in comparison to intact males [59].

It is also worth investigating the development of the syrinx, the vocal organ of birds, located in the trachea [56]. Its development seems to be strongly influenced by androgens. For example, female Japanese quails can crow when treated with testosterone - a behavior commonly exhibited only by males. This led to studying of syrinx morphology in males, females, and females treated with testosterone [62]. Unfortunately, no effect was identified on tracheal size or muscle volume; only a minor difference was observed between the right and left sides of the muscle area following testosterone treatment, with the right side being dominant. It ought to be mentioned that these data do not seem to be statistically significant, but a similar pattern has been observed previously for songbirds. For example, male and testosterone-treated female canaries show a left dominance in syrinx morphology, whereas brown-headed cowbirds and zebra finches show a right dominance [63, 64, 65, 66]. The presence of androgen receptors in the developing and adult syrinx suggests a direct effect of androgens such as testosterone on syrinx development and adult status [67, 68].

2.4.2. Androgens in avian vocalization and singing

A sound is a kind of communication signal used by many vertebrates and invertebrates. Some communicate in the form of innate vocalization others develop precise patterns by vocal learning [69].

In chicken, crowing is a typical male behavior, which is most frequently observed before dawn. Crowing is described as an innate form of vocalization since it can develop even if the auditory senses are surgically inhibited after hatch [70]. It has been well established for a long time that crowing depends on testosterone levels. Berthold

already at the end of 19th proofed the importance of testosterone not only for crowing but as well for the development of secondary sex characteristics. In this pioneering experiment, castration of immature roosters caused a reduction in male sexual behavior and prevented crowing. In contrast, after testes transplantation, subject showed typical secondary sex characteristics and aggressive behavior [71, 72].

Naturally, crowing is not observed in chicks and first appears at 10-12 weeks of age. However, several studies showed that it could be induced by testosterone administration already in very young chickens [73, 74, 75]. Marler *et al.* then analyzed sound spectrograph to distinguish strain patterns in young roosters with testosterone-induced crowing. These results showed high variability between individuals, which overrides any detectable strain differences. However, it was observed that inbred strains of chickens exhibited fewer variations than heterogeneous strains indicating the genetic background of vocalization [73]. Yazaki *et al.* reported crowing behavior after implantation of testosterone in the mesencephalon of young Japanese quails.

On the other hand, in the case of subcutaneous testosterone implantation of juvenile Japanese quails, crowing behavior was not observed. These results clearly showed that vocalization requires the development of a neural vocal system [76], which likely requires the activity of testosterone in a critical ontogenetic time window. This is even more significant in the songbirds described in the following paragraph.

In songbirds, singing is controlled by specific nuclei in the brain as the high vocal center (HVC), area X, or acropallium (RA) [77]. Nottebohm *et al.* reported that bilateral lesions of HVC in canaries caused complete degradation of the song structure. However, the birds kept their singing posture and were motivated to sing [64]. It is well documented that vocalizations or singing is sex-specific in certain species, and, in species of the Northern hemisphere it is mainly produced by males. This raises a question of hormone sensitivity and hormone regulation in birds vocal singling [77]. Vallet *et al.* reported that signing could be induced in female canaries by testosterone treatment, although this effect was not permanent [78]. Later on, it was confirmed that testosterone not only induces the singing behavior but as well causes the growth of the HVC nuclei with a structure similar to that found in males [79]. Due to the presence of androgen receptors in many parts of the song control system of songbirds, testosterone might directly affect the differentiation and activity of these areas [80, 81].

There is also a correlation between seasonal vocal activity, singing, and calling and the hormonal activity of the testicles. It has been observed that the level of sexual hormones, namely testosterone and estrogen, may influence song structure and performance in many birds. These results were compiled by Gahr [80] for different bird species. As a general trend, testosterone increases song rate independent of species, while vocal patterns differ according to species. In most species, almost all song rates were exclusively affected by the androgenic effects of testosterone and its metabolites; the

zebra finch is the only exception, where singing rates are also affected by estrogen via testosterone aromatization [80].

2.4.3. Sexual behavior

As already mentioned in chapter 2.4.2, testosterone has a high impact on sexual behaviour, aggression or secondary sex characteristics [71, 72]. In Japanese quails, a similar effect as in chickens was observed. Castration caused a reduction in mating behavior, which was restored by androgen injection [82]. Wilson and Glick studied chicken mating behavior as waltzing, attempt to mate, or mating after testosterone administration during specific time points of embryonic development. Interestingly, stimulation before embryonic day (ED) 12 did not show any significant difference compared to the untreated control. In contrast, a significant difference was observed in waltzing and mating in males stimulated at ED13, 14, and 15. Surprisingly, testosterone-induced increased waltzing behavior in females, again in case of stimulation after ED12 [83].

Circulating levels of testosterone change according to the season and affect male-male interaction, territoriality, or even female choice for mating. John Wingfield reported that testosterone implantation caused polygyny in White-crowned Sparrows. Testosterone implanted sparrows had more than one mate, and their territory was at least twice as big as by untreated birds [84]. E.g. in field study of song sparrows was confirmed that testosterone levels are at the highest point during their breeding season in the spring. After that (August to September), birds go through the molting period, testosterone is on the basal level, and gonads are partially regressed. The territorial aggression is also greatly reduced. Interestingly, castration during the off-breading season does not decrease the aggressive behavior [72].

Here, it has to be noted that also estrogen might play a role in the sexual development of males. This mechanism depends mainly on metabolic conversion (aromatization) of testosterone to estrogen in the brain. In Japanese quails, androgen aromatization was described as essential for activation of copulation [85]. Administration of aromatizable androgen to castrated zebra finches was more effective in restoring sexual and aggressive behavior than administration of androgens, which can´t be aromatized [86]. Furthermore, it was reported that conversion of testosterone to estradiol in the brain is essential for aggressive behavior in Japanese quails [87].

2.4.4. Influence of testosterone on immune system

It is well known that immune response or resistance to specific pathogens differs between the sexes. This effect is closely related to the immunomodulatory effect of sex

hormones [88]. This chapter summarize effect of androgens on the immune system of chickens.

One of the essential organs for the chicken immune system is the bursa of Fabricius, where the B-cell maturation takes place during embryonic development. It was observed that high doses of testosterone applied on three-day-old chicken embryos caused chemical bursectomy [89, 90, 91]. It is not surprising that defective development of the bursa affects B-cell functions. Hitora *et al.* reported that chickens after chemical bursectomy fail to produce antibodies against *Sallmonela pullmorum* or *Brucella abortus* [92]. Ivanyi *et al.* then analyzed the type of immunoglobulins excreted after testosterone administration against the Gumboro disease virus. The observations revealed that chicken selectively produces high levels of IgM, while the IgG production was decreased [93]. Li *et al.* then performed a challenge experiment with infection of *Sallmonela pullmorum* with or without methyltestosterone administration in young dwarf chickens [94]. The measurements showed that methyltestosterone enhanced the susceptibility to *Sallmonela pullmorum* and decreased cellular immunity [94].

In contrast, it was reported that already low doses of testosterone affect the lymphocyte population. Al-Afaleq *et al.* administrated testosterone, dihydrotestosterone (DHT), and estradiol to one-day-old chickens. This study reported a significantly decreased number of lymphocytes and, not surprisingly, hormone administration reduced the weight of the bursa of Fabricius [95]. Furthermore, estradiol and testosterone (but not DHT) decreased phagocytosis efficiency. These results indicate that testosterone or estradiol expressed immunosuppressive effect [95].

2.5. Generation of genetically modified chickens

Over the past decades, the generation of genetically modified animals became an attractive tool to analyze the function of a particular gene, understand the disease process, or find new strategies for treatment [96, 97]. Chickens are one of the most important species in poultry and a great model as a vertebrate [98, 99]. Technologies for generation of genetically modified chickens had to deal with several drawback as huge yolk or the fact that chicken egg is already laid with about 60 000 cells excluding the possibility of single cell editing [99]. In this chapter brief overview of generation of transgenic chickens is presented.

2.5.1. Gene editing via retroviral vectors

One of the first methods for the generation of transgenic animals was via retroviral vectors. Retroviral vectors possess an ability to effectively invade host cell and deliver

the recombinant DNA by random integration in the host genome. At the same, genes necessary for replication are missing ensuring that viral vectors can't regenerate infection particles [100]. The first genetically modified chicken generated by retroviral vectors was reported by Salter and his colleagues at 1987. Recombinant and wild type avian leucosis was injected into blastoderm of freshly laid eggs. Nine out of 37 males were found mosaic and exhibited germline transmission up to 11% [101].Later on further studies used viral vectors for inducing reporter gene as GFP [102], β-galactosidase [103] or interferon α-2b [104].

However, retroviral vectors bring few limitations. Essentially, size of the inserted DNA is restricted and some viral vectors exhibited high incidence of gene silencing (reduced expression) mainly after germline transmission [99]. The gene silencing was overcome by usage of lentivirus vectors. For instance, McGrew *et al.* successfully produced GFP reporter chicken by the usage of different lentivirus vectors with germline transmission from 4 to 45%. The gene silencing was not detected at all between the generation [102].

2.5.2. DNA microinjections

Microinjection is common technique for transgenesis in mammals. Nevertheless, as already mentioned in chicken, since egg is already laid in the blastoderm stage with about 50 000 to 60 000 cells, which forbid the single cell editing [100]. However, researches over the years tried to reach the single cell stage and deal with the technical difficulties. First microinjection experiment was published by Love and his colleagues. In this study, microinjection was performed into chicken egg at zygote stage. Fertilized hens were sacrificed and shell-less eggs were dissected from the oviduct. After microinjection of plasmid, were eggs cultured in the surrogate egg shell system. Seven roosters were able to reach the sexual maturity and one of them was able to transmit the exogenous DNA with efficiency of 3.4% [105]. Although, this experiment showed that by microinjection, genetically modified chicken can be successfully generated, due to the complicated set up and low efficiency, microinjections did not become widely used method in chicken transgenesis [100].

2.5.3. Cell based methods

Another approach adopted from mammalian transgenesis was usage of embryonic stem (ES) cells. Petitte *et al.* presented a somatic and germline chimera after transplantation of embryonic stem cells. ES cells from Barred Plymouth Rock chickens having a black pigment were isolated at the blastoderm stage. Then, isolated cells were injected into subgerminal cavity of Dwarf White Leghorns with white pigmentation. All of the

offspring were somatic chimeras exhibiting a mixed color of their feathers depending how efficiently the injected cells incorporated into chicken embryo [106]. Later on, the culturing conditions were characterized to be able to genetically modify chicken ES *in vitro* [107]. Unfortunately, further studies proposed that chickens produced by injection of ES can't contribute to gem-line transmission modified chickens [99, 108].

2.5.3.1. Genetically modified chickens via primordial gem cells

Nowadays, the most successful and established methods in generation of genetically modified chicken is by using primordial germ cells (PGCs). PGCs are precursors for sperms and eggs detectable at early chicken embryo in the area called germinal crescent. During the embryonic development PGCs migrate through the blood stream into the gonad, where they differentiate in functional gamets [98, 99]. Nevertheless, for long time, it was not possible to modify PGCs *in vitro*, since no culturing system was developed. Lavoir and colleagues for the first time in 2006 published a successful method of isolation, long-term culturing and re-introduction of PGCs into chicken embryos without losing the germinal competence. In this study, PGCs were isolated from Barred Plymouth Rock chickens (black pigment) and modified to express eGFP and then injected into the embryos of White Leghorn. In total, 24 male chimeric roosters were raised to sexual maturity. After breeding, all rooster were able to transfer phenotype of injected PGCs which was recognized by black feathers of the offspring. Furthermore, the eGFP expression was observed by almost 50% of the black offspring indicating the Mendelian segregation of the modified PGCs [109].

Over the following years, PGCs were used further in chicken transgenesis. Leighton *et al.* reported increased frequency on insertions of foreign DNA using phiC31 integrase mediating specific recombination between attB and attP sites [110]. Still, the integration of the foreign DNA in the chicken genome was random and no targeted gene editing was performed until then. The first precise gene editing was performed by Schusser *et al.* In this study, Ig heavy chain knock-out chicken was generated via efficient homologous recombination in PGCs [111].

Subsequently, other gene editing techniques were used for targeting in chicken PGCs. Taylor *et al.* presented knock-out of DDX4 in chicken using Transcription Activator-Like Effector Nucleases (TALENs) and homologous directed repair (HDR) [112]. TALENs are types of nucleases which can recognize 1-3 DNA basepairs specific sequence and cause a double-strand break. At the same time, homologous template sequence is provided allowing to insert foreign DNA to the target sequence [113].

Revolution in gene editing was brought by Clustered regulatory interspaced short palindromic repeats (CRISPR/Cas9) discovery as s tool for gene editing even awarded

by the Nobel Prize in Chemistry in 2020 [114, 115]. The most common type of CRISPR/-Cas9 was adapted from *Streptococcus pyogenes*. CRISPR/Cas9 consists of 3 components to aim and cause a double strand break (DBS) in the DNA. Firstly, target specific CRISPR-derived RNA (crRNA) and transactivating (tracrRNA) are joined in the form of single-chain guide RNA (sgRNA) with length 18-25 bp long. Secondly, sgRNA can localize the target, which must be followed by NGG (Protospacer adjacent motif-PAM). The third component of the CRISPR/Cas9 is Cas9 enzyme able to cause DBS 3-5 bp upstream PAM. After the DSB occurs, a repair can be done by HDR, if a suitable template is provided or by NHEJ, producing random insertions, deletions, or frameshift [113, 116].

The first transgenic chicken generated via CRISPR/Cas9 and HDR was reported by Dimitrov *et al.* In this study, loxP sites were introduced in the chicken immunoglobulin heavy chain locus in PGCs. The experiment showed that usage of CRISPR/Cas9 enormously higher the targeting efficiency [117].

3. Aims of my thesis

The scientific aim of my thesis is to analyze the role of androgens in chicken sexual development. Therefore, androgen receptor knock-out chickens were generated in order to study the effect of impaired androgen receptor/androgen signaling in male and female chickens.

Furthermore, strategies for generation of androgen receptor reporter chicken line were established.

4. Materials and methods

4.1. Animals and animal breeding

4.1.1. Chickens

The Lohmann´s Selected Leghorn Classic (LSL) was chosen in the present experiment. This line was obtained from LSL Rhein-Main Dieburg, Germany. The chickens were kept at conventional breeding conditions in the Animal Facility Thalhausen of TUM. The animal groups were raised in the aviaries size of 2 m^2. Water was supplied *ad libitum* via nipple drinkers, and the feed was specified either for young chickens or for layers later on. The experiment was approved under the license number ROB-55.2.2532.Vet_02_17-101.

4.1.2. Egg incubation

The eggs were incubated at the temperature of temperature 37.8°C, humidity 55% and were rocked three times every two hours until embryonic day 17 (ED0-17) using HEKA Favorit Olymp and Procon BSS. Then, at ED18, the rocking was stopped, and the temperature was slightly reduced to 37.2°C. During the hatch (ED20-21), the temperature was decreased further to 37.0°C, and humidity raised to 80%. During the incubation time, the eggs were candled at ED7 and ED18 to discard the infertile or dead embryos.

4.2. Cell culture methods

4.2.1. Media and supplements

This chapter describes preparation of all culturing media, which were used for different cell lines in performed experiments.

PGC medium

462.25 ml Avian KO-DMEM[1]
10 ml B27 Supplement[1]
5 ml 200mM Glutamax[1]
5 ml 10mM Non-Essential Amino Acid Solution[1]
5 ml Nukleoside 100 mM (EmbryoMAX® Nukleoside (100x))[2]
2 ml Sodium pyruvate 100x[1]
1 ml 2-Mercaptoethanol 50 mM[1]
3.75 ml Calcium chloride ($CaCl_2$) 20 mM[3]
5 ml Ovalbumin 20%
    1.2 g Ovalbumin[4]
    5 ml Avian KO-DMEM
1 ml Heparin Sulphate 50 mg/ml
    0.25 g Heparin (heparin sodium salt from porcine intestinal mucosa, TC grade)[4]
    5 ml Avian KO-DMEM

Protocol

All components were mixed under the laminar flow hood in order to keep the sterile conditions. It the end, PGC medium was filtered with 2 µm filter. The medium was stored at 50 ml falcons at 4°C. Just right before the use, the following supplements were added to the 50 ml of PGC media.

100 µl of the chicken serum[1], stored at -20°C
25 µl h-Activin-A (25 ng/µl), stored at -80°C
    200 µl Ovalbumin 0.1 % (10 ml H_2O + 50 µl Ovalbumin 20 %)
    5 µg Activin A (human Activin A)[5], stored at -80 °C
20 µl h-fibroblast growth factor $(FGF)_2$ (10 ng/µm), stored at -20°C
    25 µg bFGF (recombinant human bFGF, from *E. coli*)[7], stored at -20°C
    2.5 ml 0.1 % Bovines Serum Albumin (BSA)-solution (50 mg BSA Bovine Fraction V[4] + 50 ml PBS)

Manipulation medium

500 ml CO_2 Independent medium[1]
56.8 ml Fetal bovine serum (FBS)[4], stored at -20°C
5.7 ml Glutamax[1] , stored at -20°C

Medium for freezing cells

90 % Manipulation medium
10 % DMSO (Dimethylsulfoxid, $(CH3)_2SO$)[8], stored at room temperature

DT40 medium

500 ml Basal Iscove Medium[4]
56 ml FBS[4]
5.5 ml Chicken serum[1]
5.5 ml Glutamax[1]
1 ml 2-Mercaptoethanol[9]

Hek293T medium

500 ml RPMI/1640[4]
56 ml FBS[4]
5.5 ml Chicken serum[1]
5.5 ml Glutamax[1]

CEF medium

500 ml Basal Iscove Medium[4]
40 ml FBS[4]
10 ml Chicken serum[1]
5.5 ml Glutamax[1]

Phosphate-buffere saline (PBS), pH 7.2

8 g Sodium Choride (NaCl)[8]
1.45 g Disodium hydrogen phosphate dihydrate ($Na_2HPO_4x2H_2O$)[8]
0.2 g Potassium chloride (KCl)[8]
0.2 g Dipotassium hydrogenphosphate (KH_2PO_4)[8]
Ad 1000 ml aqua dest.
pH was adjusted to 7.2 by using 1M HCl[8] or 1M NaOH[8]

Table 4.1.: List of cell lines used in experiments.

Cell line name	Specie	Medium	Reference
PGCs	Chicken	PGC medium	[118]
Hek293T cells	Human	Hek283T medium	[119, 120, 121]
CEFs	Chicken	CEF medium	[122]
DT40 cells	DT40 medium	Chicken	[123]

4.2.2. Isolation of PGCs from chicken embryonal blood

Material

Chicken eggs
Manipulation medium
PGC medium
Mikrocappilary pipettes 40 µm[10]
DREMEL® 3000-15 Multifunctional tool[11]
Syringe Filters 0.22 µm[12]
SafeSeal 1.5 ml Cups[12]
Weight boat[14]
48-well plates[9]
Sterile scalpel[15]
Aspirator tube assemblies for calibrated microcapillary pipettes[4]
Table top incubator[13]

Protocol

The eggs were incubated under the standard conditions for 65 hours in order to reach H&H stage 14-16, at which the PGCs were isolated. The eggs were placed in the table top incubator, and one by one thin line in the middle of the egg was scored with a DREMEL® Multifunctional tool. Using the sterile scalpel, eggs were opened and placed in weight boat. Afterwards, 1 µl of the blood was drawn with microcapillary pipette and aspirator tube-system under microscope. Blood was put into the 1.5 ml SafeSeal cup with 100 µl of prewarmed PGC medium. The microcapillary pipette was always washed with manipulation medium in between the samples.

Afterwards, PGC-blood samples were transferred under the laminar flow hood in order to keep sterile conditions. At that point, the blood pellet was already visible on the bottom of the 1.5 ml SafeSeal cup of each sample. 50 µl of PGC medium were carefully removed, and the blood pellet was resuspended and transferred in 300 µl of the prewarmed PGC medium and plated on a 48-well plate. Cells were kept under standard conditions (37°C,5 % CO_2).

In the following days, cells were controlled, and if necessary, the medium was changed, or cells were passed into a new well of the 48-well plate. About 10 - 14 days post isolation, most other cell types as red blood cells died out, and PGCs started to multiplay. Afterwards, the PGCs were cultured as described in chapter 4.2.7.4 [118].

4.2.3. Isolation of PGCs from chicken embryonic gonad

Material

Chicken eggs
Manipulation medium
PGC medium
Trypsin 0.25 %[4]
PBS - BSA 0.1 %
    0.1g BSA[4]
    ad 100 ml PBS
DREMEL® 3000-15 Multifuktional tool[11]
Sterile Petri dishes[15]
24-well plates[9]
48-well plates[9]
Forceps
Table top incubator[13]

Protocol

The eggs were incubated as described in 4.1.2 for 6 - 7 fays to reach H&H stages 28 - 30, at which the PGCs were isolated. The eggs were one by one opened, and each embryo was immediately euthanized by decapitation. The torso was placed under the microscope, and embryonic gonads were gently dissected with fine forceps and placed into the 400 µl of PBS - BSA 0.1 % room temperature. Afterwards, gonad samples were transferred under the laminar flow hood, and 100 µl of the trypsin were added. The samples were digested at 37°C for 15 min.. The digestion was stopped by adding 250 µl of the manipulation media, and each sample was titrated about 20 times with the pipette to disrupt the gonad tissue. Subsequently, 300 µl of the manipulation medium were added to each sample, and samples were centrifuged at 400 xg for four min. The supernatant was discarded, and each pellet was resuspended in 350 µl of the PGC medium. Cell suspension was placed on the 24-well plates and set for two hours in the incubator (37°C,5 % CO_2). After two hours, the supernatant was carefully transferred into the 48-well plates. PGCs were then cultured as described in the chapter. 4.2.7.4.

4.2.4. Cell counting

Material

Cells
Neubauer counting chamber[15]
Trypan blue[4]

Protocol

Cells were counted prior to freezing or cell electroporation to determine cell concentrations. 10µl of the cell suspension were mixed with 10µl Trypan blue, added to the Neubauer counting chamber. Labeled squares in Figure 4.1A were counted, and cell concentration was calculated according to the equation in the Figure 4.1B.

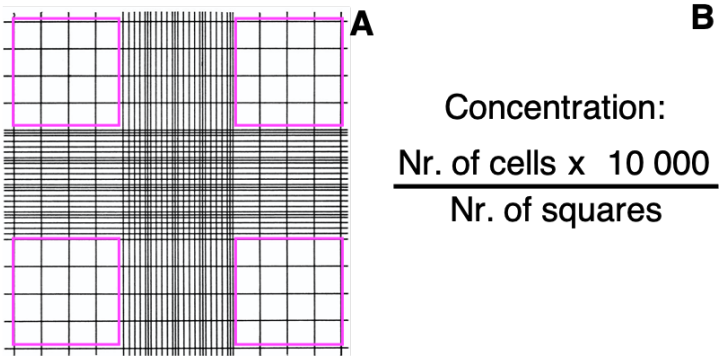

Figure 4.1.: **Scheme of the Neubauer counting chamber with the evaluation**, **A**: Neubauer counting chamber, **B**: Equation for calculation number of cell per 1 ml

4.2.5. Thawing cells

Material

Cells, stored in liquid nitrogen
Manipulation medium
50 ml falcon[12]

Protocol

Cells were transferred from the liquid nitrogen and into the water bath (37°C), where they were gently shaken until just a small ice cube was visible. Afterwards, a cold manipulation medium (20 ml, 4°C) was drop-wise added to the cell suspension, and cells were centrifuged at 300 xg for 10 min. After centrifugation, supernatant was removed, and the cell pellet was resuspended in culture media.

4.2.6. Freezing cells

Material

Cells
Manipulation medium
DMSO[8]
Cryo vials[9]
50 ml falcon[12]
Freezing box[2]

Protocol

Cells were counted and split in concentration of 1 - 2.5 10^6cells per sample. Each sample was centrifuged, supernatant was removed, and cells were resuspended in 900 µl of manipulation media. Afterwards, 900 µl manipulation media + 20% DMSO was drop-wise added to reach 10% DMSO final concentration. Cells were quickly transferred to the cryo vials and to the -80°C in freezing boxes. The next day, cells were transferred to the liquid nitrogen.

4.2.7. Cell cultivation

Cells were cultured at 37°C and with 5 % CO_2, unless specified differently.

4.2.7.1. Cultivation of DT40 cells

DT40s were passed every 48 to 72 hours. As suspension cells, DT40s were always transferred to 50 ml falcons and centrifuged at 300 xg for 10 min. (with a volume of 10 ml). The supernatant was removed, cells were diluted in DT40 media and 1/10 was transferred to a new cell culture flask.

4.2.7.2. Cultivation of chicken embryonic fibroblasts

The chicken embryonic fibroblast (CEFs) were kept at 40°C, 5 % CO_2, and were passed every 48 to 72 hours. As adherent cells, CEFs were first washed with 5 ml of PBS. Afterwards, trypsin 0.25 % was applied in order to detach cells from the surface of the culturing flask. Trypsin reaction was stopped by adding CEF culturing media. Cells were centrifuged at 300 xg for 10 min. (with a volume of 10 ml). The supernatant was removed, cells were diluted in CEF media and 1/10 was transferred a new cell culture flask.

4.2.7.3. Cultivation of Hek293t cells

Hek293T cells were passed every 48 to 72 hours. They are adherent cells, and therefore, they are cultured as described in chapter 4.2.7.2. The difference was in the culturing temperature, which was at 37°C.

4.2.7.4. Cultivation of PGCs

PGC culturing medium was finalized by adding chicken serum, activin, and FGF2 to 50 ml falcons of the PGC medium (preparation described in chapter 4.2.1). At first, cells were counted to determine the exact cell density. Afterwards, they were transferred into 50 ml falcons and centrifuged at 230 xg for 10 min. (with a volume of 10 ml). The supernatant was removed, and cell pellet was resuspended in 1/3 of the "old" conditioned culturing media, and 2/3 of fresh PGC media. PGCs were seeded in concentration 150 000 cells/ml during the week, and 100 000 cells/ml over the weekend.

4.2.8. Cell electroporation

4.2.8.1. Electroporation of DT40 and PGC

Material

$5x10^6$ DT40 cells
DT40 medium
PBS
10 mg of plasmid DNA
Cell Line Nucleofector Kit V[16]
Electroporation cuvettes (2 mm)[15]

Protocol

At first, cells were counted, and 5x10^6 were centrifuged at 300 xg for 10 min. (with a volume of 10 ml). The supernatant was removed, and cells were washed in 1 ml of PBS. Cells were transferred into 1.5ml SafeSeal Cups, and again centrifuged at 300 xg for 4 min. PBS was removed and cells were resuspended in 100 µl Cell Line Nucleofector Kit V with 10 µg of plasmid DNA. The cell+DNA suspension was transferred into electroporation cuvettes, placed in the ECM 830 Square Wave Electroporation System and electroporated using the following conditions: 8 pulses, 350 V, and 125 µsec in case of DT40. For PGC electroporation conditions were: 8 pulses, 350 V and 100 µsec. Cells were afterwards overlaid with 500 µl of the culturing media, and placed for five min. in the incubator (37°C, 5 % CO_2). Afterwards, cells were transferred in a culturing flask.

4.2.8.2. Antibiotic selection

Material

Electroporated cells
Culturing medium
- PGC medium
- DT40 medium

Antibiotic
- Puromycin (stock concentration 10 mg/ml)[1]
- Hygromycin (stock concentration 41,2 mg/ml)[8]

Protocol

In case of transient transfection, cells were 24 hours post-transfection placed into 50 ml falcon and centrifuged at 300 xg for five min. The supernatant was removed, and the cell pellet was resuspended in a medium with diluted antibiotic . After 24- or 48-hours antibiotic was removed, and cell were further cultured or analyzed. Antibiotic concentrations used for selection are shown in Table 4.2.

In case of stable transfection, cells were kept for 5 days in the incubator (37°C, 5 % CO_2). After that, antibiotic, diluted in conditioned media, was pipetted in each well. Ten days after the selection, first screening was performed. Positive clones were labeled and further observed. After another 10-14 days promising clones were gradually expanded to 24-, 12- and 6-well plates and further to T25 or T75 flasks. Antibiotic concentration was decreased during expansion to 0.75x, 0.5x and 0x.

Table 4.2.: Antibiotic concentrations used for different cell types.

Antibiotic	Cell type	Final concentration [µg/ml]
Puromycin	DT40	0.5
	PGCs	0.5
Hygromycin	DT40	2000
	PGCs	50

4.3. Androgen receptor functionality assay

Material

Plasmid DNA
Hek293T cells
ViaFect^{TM} Transfection Reagent[17]
Luciferase Assay System[17]
Opti-MEM^{TM} Serum-reduced Medium[1]
Hek293T cell medium
96-well plate[9]
96-well V-bottom plates[12]
96-well-luminescence (white) plate
Testosterone propionate (stock concentration 3.3 mM)
- 3.3 mg of testosterone propionate powder[4]
- 3 ml of ethanol for molecular biology[8]

Protocol

Hek293T cells were cultured as described in chapter 4.2.7.3. On the first day of the experiment, cells were counted and seeded in a concentration of 15 000 cells per well on the 96-well plate. The following day (24 hours), the cells were transfected with ViaFect^{TM} Transfection Reagent according to the manufacturing protocol, specific conditions are shown in Table 4.3. Cells were incubated for 24 hours and afterwards stimulated with testosterone solution (1nM). After 24-hours stimulation, cells were washed with 200 µl PBS and overlaid with lysis buffer for 10 min. Afterwards, cell lysate was harvested, transferred to 96-well V-bottom plate and centrifuged for one min.ute at 700 xg. Supernatant (10 µl) of each well was pipetted into 96-well-luminescence (white) plate and placed in the FLUOstar ® Omega plate reader. The FLUOstar ® Omega plate reader was prior to measurement set up with a substrate and adjusted in the OMEGA plate reader software. Results were exported from *MARS Data Analysis Software* and further analyzed in *GraphPad Prism 7*.

Table 4.3.: Transfection conditions used in AR functionality assay.

ViaFect^{TM} Transfection Reagent	3 µl
Plasmid 1	50 ng
Plasmid 2	50 ng
Opti-MEM^{TM} Serum-reduced Medium	Up to 50 µl per well

4.4. Semen collection from germline chimeras

Semen samples were collected from 16th week of age of the chimeric roosters to evaluate the portion of the transgene in the semen. Semen was collected 2 – 3 times a week from each chimera. Technique of collection is shown in the Figure 4.2.

The rooster was placed on the bench, one hand was holding the legs and with other the hand, an abdominal massage was given in head to tail direction. Since the gonads of the rooster are located on the back, massage gave a stimulation to release the semen in the cloacae. The second person gently squeezed the cloacae and collected the semen in 1.5 ml Eppendorf tubes.

Figure 4.2.: **Technique of sperm collection from germline chimeras**, **A**: Abdominal massage of the rooster for semen collection, **B**: Squeezing of the cloacae to collect the semen sample in the 1.5 ml Eppendort cup, adapted from [124]

4.5. Genomic DNA extraction

4.5.1. Genomic DNA extraction from cell culture cell lines

Genomic DNA from cell culture cells was extracted by using the Wizard® Genomic DNA Purification Kit or by alkaline extraction with NaOH, modified from [125].

4.5.1.1. Isolation with Wizard® Genomic DNA Purification Kit

Material

Wizard® Genomic DNA Purification Kit[17]
2.5x10^5 – 1x10^6 cells
SafeSeal Cups 1,5ml[12]

Protocol

At first, cells were centrifuged at 400 xg for four min. The supernatant was discarded, and the cell pellet was washed with 200 µl PBS. Afterwards, Wizard® Genomic DNA Purification Kit was used to extract genomic DNA according to the manufacture protocol. In the end, genomic DNA concentration was measured with NanoDrop^{TM} 1000 Spectralphotometer, and DNA was stored at -20°C.

4.5.1.2. Alkaline genomic DNA extraction with NaOH

Material

5x10^5 – 1x10^6 cells
100 mM Sodium hydroxide (NaOH)
    1 ml 1 M NaOH[8]
    ad 10 ml aqua dest.
75 mM Tris-HCl
    91 mg Tris(hydroxymethyl)-aminomethan (Tris)[8]
ad 10 ml aqua dest.
pH-value was adjusted to 7.2 with hydrochloride acid (HCl)[8]
SafeSeal Cups 1.5ml[12]

Protocol

Cells were centrifuged at 400 xg for four min. The supernatant was removed and the cell pellet was lysed with 30 µl of 100 mM NaOH. Afterwards, the cell lysate was afterwards incubated for five min. at 95°C. At the end, 100 µl of Tris-HCl were added to the mixture and samples were centrifuged at 13000 xg for 30 sec. For PCR, 1-2 µl was used as a template. DNA was stored at -20°C.

4.5.2. Genomic DNA extraction from chicken blood

Material

EDTA blood samples
TEN-buffer
- 10 mM Tris-HCl, pH 8.0[8]
- 1 mM EDTA[8]
- 10 mM NaCl[8]

STM-buffer
- 64 mM Sucrose[8]
- 20 mM Tris-HCl, pH 7.5[8]
- 10 Mm MgCl[7]
- 0,5 % Triton X-10[8]

Pronase-E
- 20 mg Pronase E[4]
- ad 1 ml H2O

96-Well mega-block plate[12]
Seal foil for 96-well plates or blocks[12]
Falcon tubes[12]
EDTA tubes[12]

Protocol

For each sample, 200 µl of STM-Buffer were prepared in a mega-block plate or in 1.5ml SafeSeal cups. One µl of the EDTA blood was added in STM-buffer. Samples were shortly vortexed and centrifuged at 1000 xg for five min. The supernatant was discarded, and pellets were resuspended in 400 µl of TEN-buffer-Pronase E (Pronase E 100 µl/ml). The mega-block plate was sealed, and samples were digested for one hour at 37°C and 250 rpm followed by inactivation at 65°C for 20 min. For PCR, 2 µl of the isolated DNA was used as a template. DNA was stored at -20°C.

4.5.3. Genomic DNA extraction from tissue

Material

Tissue samples
Lysis buffer
- 0.2 % SDS[8]
- 5 mM EDTA[8]
- 100 mM Tris-HCl, pH 8.5[8]
- 200 mM NaCl[8]

Isopropanol for molecular biology[8]
Proteinkinase K[8]
Nuclease free water[8]
96-Well Deep well plate[12]

Protocol

Lysis buffer (250 µl) and Protein kinase K (10 µl) were added to each well. A small piece of tissue was dissected and placed into the lysis buffer - Protein kinase K. The Tissue-lysis buffer solution was incubated at 56°C overnight and at 350 rpm. The next morning, the digest was shortly vortexed, and centrifuged for 20 min. and 3600 rpm. In a new deep well plate, 50 µl the isopropanol per sample were prepared for precipitation of the DNA from the tissue digest. After the centrifugation, 50 µl of the supernatant were pipetted into the isopropanol and DNA precipitates were centrifuged for 15 min. at 3600 rpm. The supernatant was discarded and pellets were air dried for 10 min. DNA precipitates were diluted by adding 100 µl of nuclease free water. If necessary, samples were incubated at 56°C for 30 min. and 350 rpm to completely desolve them. For PCR, 2 µl was used. DNA was stored at -20°C.

4.5.4. Genomic DNA extraction from chicken sperms

Material

Chicken sperm sample
TEN-buffer
- 10 mM Tris-HCl, pH 8.0[8]
- 1 mM EDTA[8]
- 10 mM NaCl[8]

Pronase-E
- 20 mg Pronase E[4]
- ad 1 ml H2O

SafeSeal Cups 1.5ml[12]

Protocol

Five µl of each sperm sample were mixed with 200 µl TEN-buffer-Pronase E solution (Pronase E 100 µl/ml). Samples were very well vortexed and incubated overnight at 37°C and 500 rpm. The next morning, samples were inactivated at 65°C for 10 min. For PCR, 1-2 µl was used as a template. The extracted DNA was stored at -20°C.

4.6. Extraction of RNA

4.6.1. Tissue homogenisation for RNA extraction

Material

Tissue samples (stored in -80°C)
InnuSpeed lysis tubes, Type P, 0.5 ml[18]
Homogenizer[18]
Dry ice
Sterile scalpel[15]

Protocol

Tissue homogenization was performed in order to extract the RNA from chicken testes. A small piece of testes (up to 30 mg) was cut with sterile scalpel. Tissue was placed in a InnuSpeed lysis tube with 200 µl of the lysis buffer mixed with thioglycerol. The testes was homogenized in the Homogenizer for 30 sec, and another 200 µl of the lysis buffer-thioglycerol were added. RNA isolation is described in chapter 4.6.2.

4.6.2. RNA extraction with ReliaPrep^{TM} RNA miniprep System

Material

Cells or homogenized tissue
ReliaPrep^{TM} RNA miniprep System[17]
SafeSeal Cups 1.5ml[12]

Protocol

RNA was isolated with ReliaPrep^{TM} RNA miniprep System either from cells or from homogenized animal tissue according to the manufacturer protocol. Concentration was measured with NanoDrop^{TM} 1000 Spectralphotometer and RNA was stored at -80°C.

4.7. Polymerase chain reaction

Polymerase chain reaction (PCR) was performed with different types of DNA polymerases. All types of PCR reactions with respective primers are described in following chapters 4.7.1- 4.7.3.

4.7.1. Polymerase chain reaction using 5xFIREPol®Master Mix

Material

DNA (0.1 ng – 10 ng)
5xFIREPol®Master Mix)[19]
Primers, forward and reverse[20,21]
Nuclease-free water[8]
SafeSeal Cups 1.5ml[12]
PCR plates[15]
Seal foil[15]
ThermoCycler

5xFIREPol®Master Mix PCR was primarily used in sex determination, RT-PCR and PCR that does not require proof-reading quality. Used primers are shown in table 4.4.

Table 4.4.: List of primers used with 5xFIREPol®Master Mix.

Nr.	Locus	Sequence (5´→ 3)	Purpose
242	cDNA AR	GACCCTGGGCAGAGGGCTACG	RT-PCR
245	cDNA AR	CAGACTGCCCAGCTTCTTCAGCTTG	
277	β - actin	TACCACAATGTACCCTGGC	Positive control for RT-PCR
278	β - actin	CTCGTCTTGTTTTATGCGC	
668	AR	GCTCTCCAGTTGCTCATCCTGAC	Loop out PCR
422	Neomycin	GCACAACAGACAATCGGCTGCTC	Loop out PCR
145	Cre	CGCTGGTTAGCACCGCAGGTG	Cre PCR
146	Cre	CCA GGT ATC TCT GAC CAG AGT C	Cre PCR

Protocol

FIREPol®Master Mix PCR was prepared according to dilution reaction mix shown below. Reaction conditions are shown in table 4.5. The annealing temperature was (table 4.4) specific for each primer pair.

Dilution for one reaction mix:

4 µl 5xFIREPol®Master Mix PCR
4 µl Forward primer (5 µM)
4 µl Reverse primer (5 µM)
variable DNA template
Up to 20 µl Nuclease-free water

Table 4.5.: 5xFIREPol®Master Mix PCR reaction conditions.

Step	Temperature	Cycle	Time
Initial Denaturation	95°C	1	5 min.
Denaturation	95°C	25-35 cycles	30 sec
Annealing	58-62°C		30 sec
Elongation	72°C		1 min./1kb
Final elongation	72°C	1	10 min.
Cool down	12°C	1	

4.7.2. Polymerase chain reaction using 5xFIREPol®MultiPlex Mix

Material

DNA
5xHOT FIREPol®MulitPlex[19]
Primers, forward and reverse[20,21]
Nuclease-free water[8]
SafeSeal Cups 1.5ml[12]
PCR plates[15]
Seal foil[15]
ThermoCycler

5xHOT FIREPol®MultiPlex PCR was used for both sex determination and for genotyping. A pool of primers was used to amplify several amplicons in one reaction. In the case of genotyping PCR, only one forward primer 668 with two reverse primers 12 and 669 were used.

Table 4.6.: List of primers used with 5xHOT FIREPol®MulitPlex .

Nr.	Locus	Sequence (5´→ 3)	Purpose
1	Z chromosome	AAGCATAGAAACAATGTGGGAC	Sex determination
2	Z chromosome	AACTCTGTCTGGAAGGACTT	
3	W chromosome	CTATGCCTACCACMTTCCTATTTGC	
4	W chromosome	AGCTGGAYTTCAGWSCATCTTCT	
12	Neomycin	AGTGACAACGTCGAGCACAGCT	ARKO Genotyping
668	AR	GCTCTCCAGTTGCTCATCCTGAC	
669	AR	GTCAGACTGCTCTGCTGGAG	

Protocol

5xHOT FIREPol®MulitPlex PCR was prepared according to dilution reaction mix shown below. Reaction conditions are shown in Table 4.7.

Dilution for one reaction mix:

4 µl 5xFIREPol®MultiPlex
4 µl Forward primer (5 µM)
4 µl Forward primer (5 µM)
4 µl Reverse primer (5 µM)
4 µl Reverse primer (5 µM)
variable DNA template
Up to 20 µl Nuclease-free water

Table 4.7.: 5xFIREPol®MultiPlex Mix PCR reaction conditions.

Step	Temperature	Cycle	Time
Initial Denaturation	95°C	1	12 min.
Denaturation	95°C	25-35 cycles	30 sec
Annealing	58-62°C		30 sec
Elongation	72°C		1 min./1kb
Final elongation	72°C	1	10 min.
Cool down	12°C	1	

4.7.3. Polymerase chain reaction using Q5® High-Fidelity DNA Polymerase

Material

DNA (10pg - 10ng)
Q5® High-Fidelity DNA Polymerase[6]
5xQ5 Reaction Buffer[6]

dNTPs[1]
Primers, forward and reverse[20,21]
Nuclease-free water[8]
SafeSeal Cups 1.5ml[19]
PCR plates[15]
Seal foil[15]
ThermoCycler

Q5® High-Fidelity DNA Polymerase PCR was performed to amplify DNA fragments used for Gibson Assembly (GA), since the Q5® High-Fidelity DNA Polymerase obtains proof-reading characteristics and provides high quality of the amplified fragments. Primer list is displayed in the table 4.8 below.

Table 4.8.: List of primers used with Q5® High-Fidelity DNA Polymerase .

Nr.	Locus	Sequence ($5´ \rightarrow 3$)	Purpose
339	AR	CACTATAGGGCGAATTGGGTACCGGCGC GCCGTTGCACAGTCTCCCTGTTTTTG	Gibson Assembly of #116
340	AR	ATAATGTATGCTATACGAAGTTAT GCTAGCTGCAGAGAGAAGAGAGC	
341	AR	TACCCCAGTTGGGGCACACACG TCACTAGTAACGCACACAGAGAGG	
342	AR	AAAGCTGGAGCTCCACCGCGGTGG CGGCCGCACCTTTCCTAAGCTAATGAC	
858	AR-codon-optimized	CCACTAGTCCAGTGTGGTGGAATTCCG CCACCATGGAGGTTCAACTCGGTATCGG	Gibson Assembly of #247,#260,#261
859	AR-codon-optimized	CGGGCCCTCTAGACTCGAGCGGCCGC TCATTTATCGTCATCGTCTTTGTAATCC TCGGCATGGAAGTAAATTG	
860	AR-codon-optimized	CGGGCCCTCTAGACTCGAGCGGCCGC TCATTTATCGTCATCGTCTTTGTAATC AGAACCACCAGAACCACC CTCGGCATGGAAGTAAATTG	
929	mCherry	ATTTACTTCCATGCCGAGGGTGGTT CTGGTGGTTCTGTGAGCAAGGGCGAGGAG	
930	mCherry	CTCCTCGCCCTTGCTCACAGAACCACC AGAACCACCCTCGGCATGGAAGTAAAT	
931	AR-mCherry	CGGGCCCTCTAGACTCGAGCGCGGCCG CTCACTTGTACAGCTCGTC	

Protocol

5® High-Fidelity DNA Polymerase PCR was prepared according to dilution reaction mix shown below. Reaction conditions are shown in table 4.9. For each primer pair, the specific annealing temperature was used.

Dilution for one reaction mix:

5 µl 5xQ5 Reaction Buffer
0.5 µl dNTP
1.25 µl Forward primer (0.3 µM)
1.25 µl Reverse primer (0.3 µM)
0.25 µl Q5® High-Fidelity DNA Polymerase
5 µl 5xQ5 High GC Enhancer (optional)
variable DNA template
Up to 25 µl Nuclease-free water

Table 4.9.: Q5® High-Fidelity DNA Polymerase PCR reaction conditions.

Step	Temperature	Cycle	Time
Initial Denaturation	98°C	1	30 sec
Denaturation	98°C	25-35 cycles	10 sec
Annealing	50-72°C		30 sec
Elongation	72°C		20-30 sec/1kb
Final elongation	72°C	1	2 min.
Cool down	12°C	1	

4.8. Restriction enzyme digest

Material

Restriction enzyme[6]
Enzyme specific restriction buffer[6]
Nuclease free water[8]
Plasmid DNA

Protocol

Restriction enzymes, naturally occurring in bacteria, are able to cut specific site of DNA sequences. For that, the restriction digest was set up before the cloning of the DNA fragment by Gibson Assembly (4.12.2) or before cell electroporation (4.2.8) to linearize the electroporated construct.

The sequence was analysed in software *Lasergene Seqbuider* (DNASTAR, Madison, USA) or with an online platform Benchling available at www.benchling.com. The digest was set up as shown in table 4.10. Result of the restriction digest was analyzed by gel electrophoresis (table 4.10).

Table 4.10.: Dilution of restriction digest.

Restriction enzyme 1	5 units
Restriction enzyme 2 (optional)	5 units
Enzyme specific buffer 10x	2 µl
Nuclease free water	Ad 20 µl

4.9. DNA precipitation with sodium acetate

Material

Ethanol for molecular biology[8]
Sodium acetate 3 M (CH_3COONa)
2.4 g Sodium acetate anhydrous for molecular biology[8]
Up to 10 ml of double distilled water
Nuclease free water[8]
Sterile PBS

Protocol

DNA precipitation was performed to concentrate DNA either after restriction digest (4.8) or after plasmid isolation from a bacterial culture (4.12.5). Initial volume of DNA precipitate was measured, and a 1 to 1 volume ratio of 100 % ethanol was added. Subsequently, CH_3COONa (1/10 of the DNA/Ethanol mix) was mixed thoroughly with the DNA-ethanol solution. Precipitated DNA was centrifuged at 16 000 xg for 10 min. at 4°C. The supernatant was removed and DNA pellet was washed by adding 500 µl 75 % ethanol, centrifuged at 14 000 xg for 10 min. at 4°C. The supernatant was discarded, and the pellet was air dried for 10-30 min. at room temperature. DNA precipitate was diluted in preheated PBS (50°C) to the concentration of 1µg/µl. If necessary, the pellet was heated for additional 10 min. at 50°C.

4.10. Gel Electrophoresis

Material

Agarose low EEO[8]
10x Tris-Borate-EDTA buffer (TBE buffer)
108 g Tris(hydroxymethyl)-aminomethal (Tris)[8]
55 g Boric acid[8]
40 ml 0.5M EDTA, pH 8.0[8]
Ad 1000 ml aqua dest.
1x Tris-Borate-EDTA buffer (TBE buffer)
1:10 dilution of 10xTBE buffer with aqua dest.
DNA/RNA dye, peqGREEN[15]
6x DNA Loading dye
60 ml Glycerol[8]
12 ml 0.5M EDTA, pH 8.0[8]
100 mg Orange G[4]
Ad 100ml aqua dest.
DNA Ladder
4.5 µl DNA 1kb Plus DNA Ladder[6]
120.5 µl Nuclease-free water[8]
25 µl 6x DNA Loading dye

Protocol

Gel electrophoresis was performed to visualize the outcome of the PCR (4.7) or the restriction digest (4.8). For this purpose, agarose gel was prepared using a w/v solution in the 0.5-1% range, optimized for the size of the analyzed DNA fragment. The optimal percentage of the agarose gel results in the best separation and resolution of DNA fragments.

Agarose powder was mixed with 1x TBE buffer and heated up in the microwave until the powder was dissolved. After that, the mixture was cooled down in the water bath, and peqGREEN DNA (4 µl per 100 ml) was added for DNA visualization. The liquid gel mixture was poured into the gel tray with a comb. The dried gel was transferred to the electrophorese chamber and overlaid with TEB buffer.

DNA samples were premixed with 6x DNA Loading dye (1x final concentration) and 10 µl of each sample were loaded on the gel. 8 µl of the DNA Ladder were loaded on the gel next to the sample to be able to determine the DNA fragment size. Electrophorese chamber was connected to the electric source and gel was run according to its size at 80 - 220 V for 60 - 90 min.

The DNA was detected using a UV-light gel documentation system (Quantum, Vilber Lourmat). Gel pictures were exported and further processed in Microsoft PowerPoint.

4.11. Extraction of DNA from gel

Material

50x Tris-Acetat-EDTA buffer (TAE buffer)[1]
1x TAE-Puffer
    1:50 dilution of 50x TAE buffer
E.Z.N.A® Ultra-Sep® Gel Extraction Kit[15]
Sterile scalpel[15]
BlueLight Table (Serva)

Protocol

Gel extraction was performed to purify DNA either for Gibson Assembly or for sequencing.

The gel was prepared as described in chapter 4.10, only TAE buffer instead of TBE buffer was used. After 60-90 min. of separation in the electrophoretic chamber, the gel was transferred to a BlueLight Table (Serva), and the band of the correct size was cut out with a scalpel. Weight of the gel piece was measured, and from now on DNA was extracted with E.Z.N.A® Ultra-Sep® Gel Extraction Kit according to the manufacturer protocol.

4.12. Vectors and cloning

4.12.1. List of constructs and ssDNA

Tables Table 4.11 and Table 4.12 summarize initial constructs used for further cloning as well as construct used for cell transfection.

Table 4.11.: List of original constructs used for cloning.

Construct number	Vector	Antibiotic resistance prokaryotic/eukaryotic	Source
#34	PX330-U6-Chimeric-BB-CBh-hSpCa9	Ampicillin	Feng Zhang (Addgene plasmid #42230)
#38	pSpCas9(BB)-2A-GFP (PX458) V2.0	Ampicilin/Puromycin	Feng Zhang (Addgene plasmid #48138)
#39	pSpCas9(BB)-2A-Puro (PX459) V2.0	Ampicilin/Puromycin	Feng Zhang (Addgene plasmid #62988)
#73	Bluescript	Ampicilin/Puromycin	Prof. Dr. Benjamin Schusser, Reproductive Biotechnology, TUM School of Life Sciences, TU Munich
#154	pcDNA3.1-Flag-FoxP3	Ampicilin/Neomycin	Prof. Dr. Benjamin Schusser, Reproductive Biotechnology, TUM School of Life Sciences, TU Munich
#246	pGL4.36[luc2P/MMTV/Hygro]	Ampicilin/Hygromycin	Promega, E1360

Table 4.12.: List of generated constructs used for cell transfection.

Construct number	Vector	Original construct	Insert	Restriction sites	Primers
#116	Bluesript	#73	ARKO-Repair eGFP-Puro	SpeI, NotI and AcsI, NheI	339-342
#125	px330	#34	sgRNA1429-px330-ARKO	BbSI	403,404
#205	px458	#38	sgRNA1432-px458-ARKI	BbSI	495,496
#206	px459	#39	sgRNA1432-px459-ARKI	BbSI	495,496
#247	pcDNA3.1	#154	pcDNA3.1-AR-codon-optimized	EcoRI,XBaI	
#260	pcDNA3.1	#154	pcDNA3.1-AR-codon-optimized-FLAG	EcoRI,XBaI	858,859
#261	pcDNA3.1	#154	pcDNA3.1-AR-codon-optimized-GC-FLAG	EcoRI,XBaI	858,860
#362	pcDNA3.1	#154	pcDNA3.1-AR-codon-optimized-GS-mCherry	EcoRI,XBaI	858,929-931

4.12.2. Gibson Assembly

The Gibson Assembly allows an assembly of multiple linear DNA fragments with the backbone of the vector. In all experiments, DNA fragments were amplified Q5® High-Fidelity DNA Polymerase using primers shown in table 4.8. For the primer design, the online tool NEBCloner® [126] was used, and primers obtained 18-30 bp overhangs to the backbone of the vector.

Material

NEBuilder® HiFi DNA Assembly Master Mix[6]
DNA fragments
Vector backbone
Nuclease-free water[8]

Protocol

The Gibson assembly reaction mix was always set up on ice. Herein, 2 - 3 fragments were assembled with the vector backbone at the same time. All components shown in table 4.13, were mixed, and reaction was incubated in a thermocycler for one hour at 50°C. In the end, 2 µl of the reaction were used for bacterial transformation as described in chapter 4.12.4.

Table 4.13.: Gibson Assembly reaction mixture.

2–3 Fragment Assembly	Molar ratio backbone:insert 1:2
Total Amount of Fragments	X µl (0.03–0.2 pmols)
NEBuilder® HiFi DNA Assembly Master Mix	10 µl
Nuclease-free water	Up to 20µl

4.12.3. CRISPR/Cas9 Cloning

Material

Cloning vector px330, px458 or px459
sgRNA oligos[20,21]
10x T4 Polynucleotide Kinase (PNK) buffer[6]
T4 PNK[6]
10x Tango buffer[1]
Dithiothreitol (DTT)[8]
Adenosine triphosphate (ATP)[1]

FastDigest BbsI[1]
T7 ligase[6]
10x PlasmidSafe Buffer[24]
PlasmidSafe exonuclease[24]

In our experiments, sgRNAs were designed with an online platform Benching available at (https://www.benchling.com). sgRNAs were cloned in the CRIPSR/Cas9 vector using the protocol described by Cong *et al.* [127]. All sgRNA oligos were synthesized with the following overhangs.

5′ – CACCGNNNNNNNNNNNNNNNNNN – 3′
5′ – CNNNNNNNNNNNNNNNNNNCAAA – 3′

Table 4.14 displays primers designed for sgRNA cloning into the CRISPR/Cas9 vectors.

Table 4.14.: List of sgRNA used for cloning of CRISPR/Cas9 vectors.

Nr.	Locus	Sequence (5´→ 3)	Purpose
gRNA1429	Exon 2 of AR	CACCGgcaaagtgttcttcaagcggg	Cloning of #125 for ARKO
gRNA1429	Exon 2 of AR	AAACcccgcttgaagaacactttgcC	
sgRNA1432	Exon 8 of AR	CACCGgcacgcggagtgatcttctgg	Cloning of #205 and #206 for ARKI
sgRNA1432	Exon 8 of AR	AAACccagaagatcactccgcgtgcC	

Protocol

1. Phosphorylation and annealing of sgRNA oligos

   1 µl top sgRNA oligo
   1 µl bottom sgRNA oligo
   1 µl 10x T4 PNK buffer
   0.5 µl T4 PNK
   6.5 µl Nuclease-free water

   All components were mixed in a PCR tube, and subsequently placed into the thermocycler for 30 min. at 37°C, followed by five min. incubation at 95°C. After that, reaction mixture was steadily cooled down to 25°C (decrease 5°C per minute). Final annealed sgRNA product was diluted 1:250 in nuclease-free water.

2. Digestion and ligation reaction

   100 ng px330
   2 µl Diluted annealed sgRNA oligos from step 1
   2 µl 10x Tango buffer
   1 µl DTT
   1 µl ATP
   1 µl FastDigest BbsI
   0.5 µl T7 DNA ligase
   Up to 20 µl Nuclease-free water

   The reaction mixture was set up, and incubated in the thermocycler unde the following conditions:

   37°C for 5 min.
   23°C for 5 min.
   Both steps in total of six cycles.

3. Treatment with PlasmidSafe exonuclease to prevent unwanted recombination

   11 µl ligation product from step 2
   1.5 µl 10x PlasmidSafe buffer
   1.5 µl 10 mM ATP
   1 µl PlasmidSafe exonuclease

All components were mixed, and the reaction mixture was incubated for 30 min. at 37°C. Final product was then transformed in bacteria, as described in chapter 4.12.4. The success of the cloning was verified by restriction digest and sequencing.

4.12.4. Bacterial transformation

Material

Competent bacteria (E. coli, DH5 α)[6]
    Stored at -80°C
Super optimal broth with catabolite repression-medium (SOC-medium)[6]
Lysogeny Broth (LB)-Ampicillin-Agar plates
    Ampicillin (1:1000 dilution of 100µg/ml)

Protocol

The chemical competent bacteria were taken from a -80°C freezer and kept for five min. on ice. After that, two µl of the cloning product (4.12.2 or 4.12.3) were pipetted on the bacterial suspension, and incubated for 30 min. on ice. Afterwards, the heat shock of the bacterial suspension was performed at 42°C for 30 sec., followed by cooling down on ice for two min. At the end, 950 µl of the SOC-medium were added to the bacteria and shaken at 37°C for 60 min. The bacteria-SOC media suspension was spread on LB-Agar plates and placed in an incubator (37°C) overnight. After 12-16 hours, plates were removed from the incubator and checked for colonies.

4.12.5. Isolation of plasmid DNA from bacterial culture

Material

PureYield^{TM} Plasmid miniprep System[17] or
PureYield^{TM} Plasmid Midiprep System[17]
LB medium
- 25 g LB medium[6]
- Up to 1000ml aqua dest.
- Stored at 4°C

LB-ampicillin medium
- Ampicillin (1:1000 dilution of 100µg/ml)
- Stored at 4°C

Protocol

Bacterial colonies (4.12.4) were picked with a pipette tip and placed in 5 ml of LB-ampicillin media. Bacterial culture was shaken overnight at 37°C and 250 rpm. Next day, in the morning, plasmids were isolated with PureYield^{TM} Plasmid miniprep System in line with the manufacturer protocol.

For preparation of larger amounts of plasmid, necessary for cell transfection, 5 ml of the bacterial culture wasset up during the day as a starter culture. After 6-8 hours of shaking, the day culture was poured into 250 ml of LB-ampicillin media, and shaken overnight. Plasmid isolation was performed by PureYield^{TM} Plasmid Midiprep System according to the manufacturing protocol.

4.13. Generation of germline chimera

Material

PGCs
Chicken eggs (LSL Classic strain)[22]
Turkey eggs for surrogates
Eggs white
Mamipulation medium
Sterile de-ionized water
Mikrocappilary pipettes 40 µm[10]
DREMEL® 3000-15 Multifunctional tool[11]
Penicilin-Streptomycin solution (Penicilin 100 IU/ml and Steptomycin 100 µm/ml)[4]
Syringe Filters 0.22 µm[12]
SafeSeal 1.5 ml Cups[12]
Weight boat[14]
Sterile scalpel[15]
Aspirator tube assemblies for calibrated microcapillary pipettes[4]
Wrap foil Saran^{TM} Clin Plus[23]
Table top incubator

4.13.1. Intravenous injection of PGCs

The intravenous injection was performed at Hamburger & Hamilton stages 14-16. The eggs were briefly incubated for 65 hours. The eggs were carefully opened without breaking the egg yolk. For that, a very thin line was scored in the eggshell with the DREMEL® 3000-15 multifunctional tool with one deeper cut, as displayed in Figure 4.3 A. With the scalpel, the egg was gently opened (Figure 4.3 B) and the chicken embryo was then placed in the weight boat (Figure 4.3 C, D). The embryo was then transferred under the microscope, where the intravenous injection of the PGCs was performed. 1 µl of the PGCs suspension was taken, and injected in dorsal aorta using a 40 µm microcapillary pipette (3000 cells/µl in manipulation media). After each injection, microcappilary pipette was washed with filtered manipulation media. During the whole procedure, the eggs were kept in the tabletop incubator at 37°C.

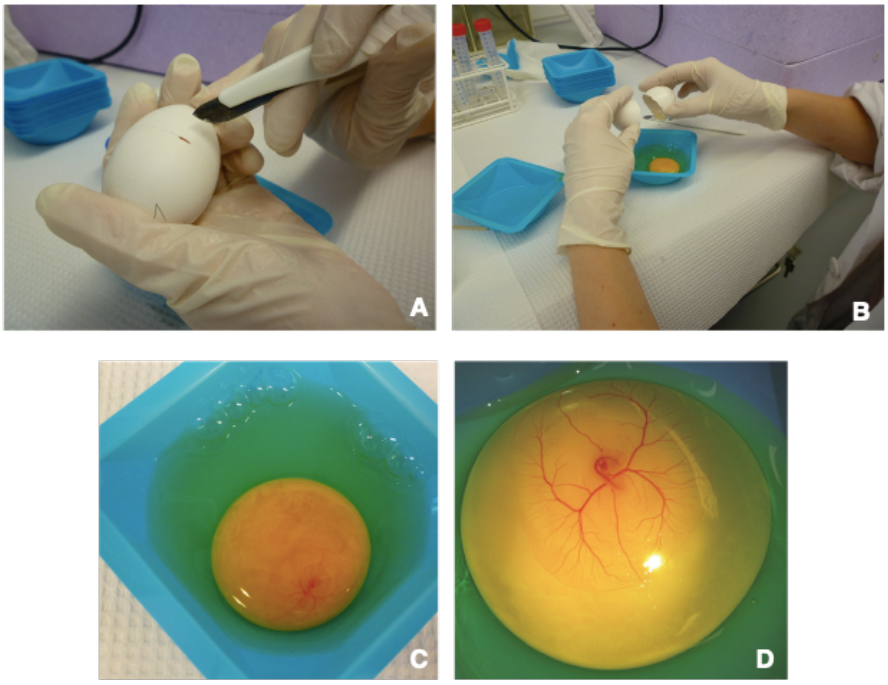

Figure 4.3.: **Technique of egg opening prior the intravenous injection of PGCs**, **A**: Egg scored by using DREMEL® 3000-15 multifunctional tool, **B**: Gentle opening of the chicken egg in the weight boat, **C**: Chicken embryo in the weight boat, **D**: Chicken embryo in the weight boat displaying the heart and vessels, adapted from [124]

4.13.2. Transfer to the surrogate egg shell

Since the chicken egg shell got lost via the opening process, turkey eggs were prepared as surrogate egg shell for injected chicken embryos. It was necessary that weight of surrogate egg was 30-40 g bigger than the weight of the chicken egg. Injected embryos were poured in the surrogate eggshell (Figure 4.4 C). Surrogate eggs were closed with the wrapping foil. In order to seal the egg shell completely, and to prevent contamination and drying, wrapping foil was sealed with albumin, mixed with Penicilin-Steptomycin solution (in dilution 1 to 10). (Figure 4.4 D-F).

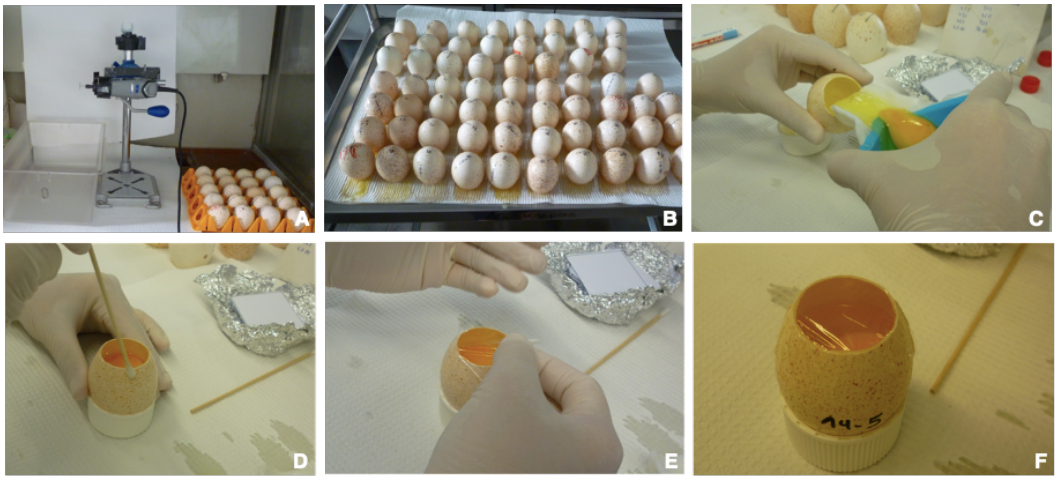

Figure 4.4.: **Turkey egg surrogate system**, **A, B**: Turkey egg surrogate preparation, **C**: Pouring of the chicken embryo in the turkey egg shell surrogate, **D-F**: Sealing the surrogate egg shell with the egg white-Pen/Step solution and the wrap foil, adapted from [124]

The injected chicken embryos were kept in the incubator until the day of the hatch. At ED18, the rocking was switched off. During the hatching process, chickens were controlled every 4-6 hours. The process was assisted until successful hatch of the chimeras (Figure 4.5 A-C).

Figure 4.5.: **Hatching of the germline chimera**, **A-C**: Hatching process of the chicken embryo from the surrogate egg shell, adapted from [124]

4.13.3. Testing of germline transmission

Male chimeras, hatched from turkey egg surrogate eggs, were raised to sexual maturity. From the 16th week of age, semen of the germline chimeras was collected and tested via PCR for the genetic modification, as described in chapter 4.4. Promising candidates, tested at least three times positive for the genetic modification were bred to WT hens. In order to prove germline transmission, transfer of the genetic modification to the next generation, eggs from the chimera breeding were collected for three weeks and incubated. At ED7, each egg was opened, and either checked for GFP expression or a small piece of tissue was taken to be analyzed via PCR for the presence of the genetic modification. In case of positive germline transmission, eggs from this breeding were further collected and incubated until hatch.

4.14. Testosterone induced bursectomy

Material

Cryo vials[9]
Dry ice
Ice
1% Testosterone solution
1 g of testosterone propionate powder[4]
EtOH for molecular biology[8]

Protocol

The eggs were set under the standard conditions. At ED3, eggs were removed from the incubator and dipped in the ice cold 1% testosterone solution. Each egg was dipped about 3.5 cm from the pointy end. Eggs were then incubated until ED18. At ED18,

embryos were sacrificed and bursa of Fabricius was dissected and frozen down. Organs were kept at -80° C until cryo-sectioning.

4.15. Histology

4.15.1. Organ sampling and cryosectioning

Material

Cryo vials[9]
Dry ice
Object slides, Superfrost5® Plus[15]
Microsome blades (Feather®)[5]
Tissue-Tec®[5]
Paint brush

Protocol

The organs were taken at certain time points, placed in the cryo tubes and frozen on dry ice. Until cryo-sectioning, the organs were stored in -80° C.

The day before cryo-sectioning, samples were placed at -20° C to allow the temperature to adjust. On the day of cryo-sectioning, the organs were transferred in the microtome chamber and fixed with Tissue-Tec®on specimen chucks. The organs were cut at 4 µm and each section was transferred to Superfrost5®Plus object slides. Slides were then air dried overnight.

4.15.2. Immunohistochemical staining

Material

Aceton 100%[2]
PBS (See 4.2.1)
Bovine serum albumin (BSA)[8]
Horse serum[25]
Methanol 40%[8]
Hydrogen peroxide 30% (H_2O_2)
Primary antibody #21 (See 4.15) [25]
Secondary biotin-conjugated antibody[15]
Vector® DAB kit[25]

Vectastatin® ABC kit[25]
Eukitt® mounting medium[4]
Mayer´s Hematoxylin solution[26]
Aqua dest.
Ascending alcohol row[2]
- Ethanol 70%
- Ethanol 99%
- Isopropanol I
- Isopropanol II
- Xylol I
- Xylol II

Parafilm[8]
Incubating chamber
Cover slips[15]

Protocol

Sections were first fixed in ice-cold acetone for two min. Sections were then air-dried for 10 min. and afterwards rehydrated for 15 min. in PBS. In order to block the activity of the endogenous peroxidase, samples were overlaid with H_2O_2-methanol solution (in dilution 1 to 100), covered with parafilm and incubated for 30 min. in the dark at room temperature. Subsequently, sections were washed three times for five min. in PBS. For prevention of any non-specific antibody binding, samples were blocked with horse serum diluted 1 to 40 in 1% PBS-BSA solution. Sections were incubated for one hour at room temperature in an humidified chamber. Afterwards, the primary antibody was incubated on the sections at room temperature for one hour or at 4° C over night. The primary antibody was then washed off three times for five min. with PBS. Secondary antibody staining was performed according to manufacturer protocol of Vectastatin® ABC kit. Peroxidase detection was performed with Vector® DAB kit. Staining of the nuclei was done by incubation of the sections in Mayer´s Hematoxylin solution for one minute. Nuclei staining was stopped by running tap water for five min. The last dehydration step was performed by using an ascending alcohol row as it is shown in the material section. On each slide 1 drop of Eukitt® mounting medium was applied, and sections were covered with coverslips. Samples were air-dried overnight.

4.16. Fluorescent activated cell sorting (FACS)

4.16.1. Isolation of leukocytes from chicken blood

Material

PBS (See 4.2.1)
Biocoll Separating Solution[4]
Ice
FACS tubes[12]

Protocol

Blood was collected from chickens at 3, 6 and 12 week of age to EDTA tubes. EDTA-blood was then diluted 1 to 1 with PBS and slowly overlaid on Biocoll separating solution prepared in 5 ml FACS tubes. Lymphocytes were separated by density gradient centrifugation for 12 min. at 650 xg on with slow acceleration and no breaks. After the centrifugation step, lymphocytes were isolated from the interface between plasma and Biocoll separating solution. At the same time, plasma was collected. The cells were further washed with PBS and stained as described in chapter (4.16.2).

4.16.2. Staining and measurements

Material

Leucocytes
PBS (See 4.2.1)
Fluo buffer
    5g Bovine Serum Albumin - Fraction V9[8]
    50 mg Sodium azide (NaN_3)[8]
    Ad 500 ml PBS
Primary and secondary antibodies (See 4.15 and 4.16), dilution in fluo buffer
Fixable Viability Dye eFluor 780[1], dilution in fluo buffer
Ice
96-well plates, U-bottom[12]

Protocol

Isolated lymphocytes were counted, diluted to 500 000 cells/well and plated on 96 well U-bottom plates. Each sample was resuspended in 200 µl of fluo buffer and centrifuged for one min. at 700 xg. The supernatant was discarded and cells were stained with 50 µl Fixable Viability Dye eFluor 780 (in dilution of 1:1000) for discrimination of living cells (incubation for 20 min. on ice). The live/dead staining was washed away by adding 200 µl fluo buffer and centrifugation for one min. at 700 xg. The supernatant was discarded and the primary antibody (Table 4.15) was diluted in 50 µl of fluo buffer and applied to the cell pellet. Cells were incubated with a primary antibody for 20 min. on ice. Washing was performed as in previous steps. The secondary antibody

staining was applied on cells (diluted in 50 µl of fluo buffer, Table 4.16) and incubated for 20 min. on ice. After the incubation time, cells were washed (700 xg, one min.) and the pellet was resuspended in 300 µl of fluo buffer. Samples were measured using an *Attunes NxT* FACS. Data was analyzed using the software *FlowJo10*.

4.17. Antibodies used for imunohistochemical staining and FACS

Table 4.15.: List of primary antibodies used for immunohistochemical staining and FACS.

Number	Antigen	Clone	Host	Conjugate	Isotype	Working concentration	Supplier
18	chγδ-TCR	TCR1	Mouse	UNLB	IgG1	2.5 µg/ml	25
19	chαβ-TCR	TCR2	Mouse	UNLB	IgG1	2.5 µg/ml	25
20	chαβ-TCR	TCR3	Mouse	UNLB	IgG1	2.5 µg/ml	25
21	Bu1(a+b)	AV20	Mouse	UNLB	IgG1	5 µg/ml	25
23	chMQ	Kul01	Mouse	UNLB	IgG1	5 µg/ml	25

Table 4.16.: List of secondary antibodies used for immunohistochemical staining and FACS.

Number	Antigen	Clone	Host	Conjugate	Isotype	Working concentration	Supplier
26	Anti-Mouse IgG(H+L)		Goat	APC	IgG1	2.5 µg/ml	25

4.18. Statistical analysis and graphs

The statistical analysis was performed in R-studio software running on R version 4.0.3. At first, data were analyzed with a Shapiro-Wilk test of normal distribution. Secondly, variances of the analyzed groups were compared with a F-test. Then, a non-parametric two sample t-test was performed with a level of significance on 0.05. An example of the R script is shown in Appendix section

The graphs were designed in GaphPad software.

5. Results

5.1. Generation of androgen receptor knock-out (ARKO) chicken

In this chapter, the process of the generation of androgen receptor knock-out chickens is described. At first, a targeting construct (pBluescript) with two homologous arms was cloned and afterwards, two sgRNA were tested for a knock-out of exon 2 of the androgen receptor (AR). sgRNA1429 was chosen and together with targeting construct transfected into chicken primordial germ cells (PGCs). Clonal ARKO PGCs were injected into chicken embryos to produce germline chimeras via the surrogate egg shell system. Then, male hatched chickens were raised to sexual maturity, and their semen was tested for a transgene. Promising candidates were bred to WT hens to produce ARKO heterozygous, and later on, to ARKO homozygous chicken. The strategy for the ARKO is displayed in the Figure 5.1.

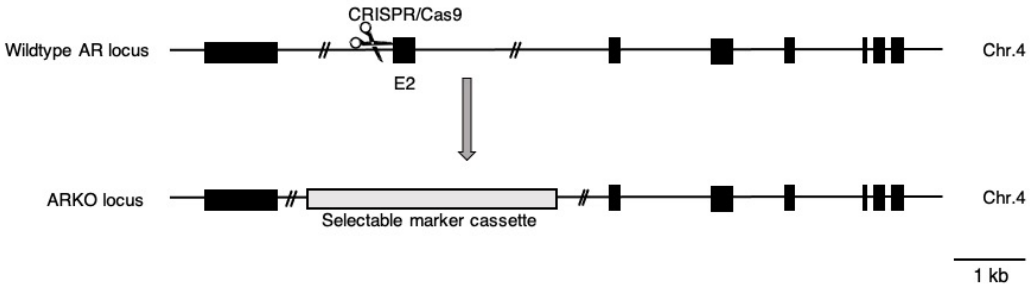

Figure 5.1.: **Androgen receptor knock-out strategy**, exon 2 of AR was knocked-out using CRISPR/Cas9 system. Instead of exon 2, a selectable marker cassette was inserted allowing to discriminate successfully edited PGCs clones.

5.1.1. Cloning of the ARKO repair construct for editing in PGCs

For the androgen receptor knockout (ARKO), at first a targeting construct was cloned. The constructs back-bone contained a selectable marker cassette consisting of eGFP, expressed under the chicken β-actin promoter, and a gene for puromycin resistance,

expressed under the CAG promoter. eGFP served as a marker for germline transmission in future breeding, and puromycin-resistant-gene enabled the selection of primordial germ cells with successful editing. The cassette was flanked by HS4 insulators and loxP sites on its 5′ and 3′ site. LoxP can be recognized by Cre recombinase, allowing the loop out of the selectable marker cassette. Additionally, the backbone contained an attB70 sequence as a recognition site for phiC31 integrase and a promoter less gene for neomycin resistance.

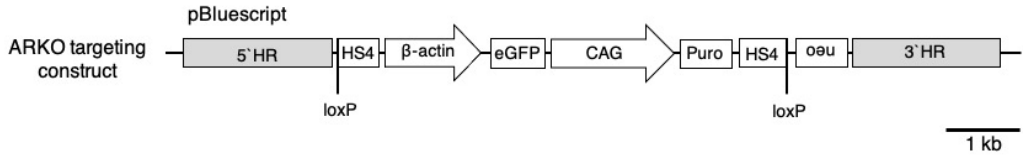

Figure 5.2.: **Scheme of the ARKO targeting construct**, pBluescript vector backbone was used for the cloning of a ARKO targeting construct. The ARKO targeting construct contained two homologous arms on 5′ and 3′ and selectable marker cassette. The selectable marker cassette included a puromycin resistant gene expressed under the CAG promoter and eGFP expressed under the chicken β-actin promotor. Furthermore, the construct contained 5′ and 3′site HS4 insulators and a loxP site enabling the loop out of the selectable marker cassette.

The selectable marker cassette was flanked by two homologous arms (HR) on the 5′ and the 3′ site. Homologous arms were designed to target exon 2 of the AR. The sequence of the homologous arms was amplified from gDNA of PGC-LSL line 2-6 via PCR using specific primers. The PCR was run in four reactions, which were pooled and the DNA was concentrated via gel extraction. The PCR result is displayed in the Figure 5.3 showing the fragment of the correct size of 2200bp for 5′HR and 2300bp 3′HR.

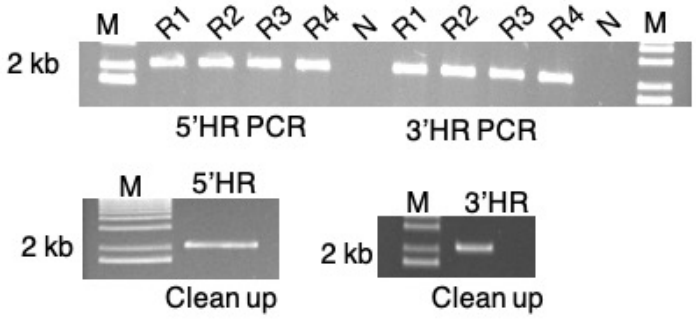

Figure 5.3.: **PCR for amplification 5'HR and 3'HR**, PCR was always prepared in four reactions giving a sufficient amount of DNA for further cloning. Reactions having correct fragment size on the gel electrophoresis were pooled and purified via gel extraction, M = marker, R = Reaction.

The cloning of each HR was verified by a restriction digest and by sequencing as displayed in the Figure 5.4. For 5´HR, all picked clones exhibited a fragment of the expected size on the control digest and therefore, one clone was sent for sequencing. 5´HR of sequence successfully aligned to the template. For 3´HR, three out of four clones showed the expected fragment size on the restriction digest. Again, one clone was sent for sequencing, which confirmed the presence of 3´HR after aligning to template. The construct was inserted in the internal lab database and was entitled as construct #116.

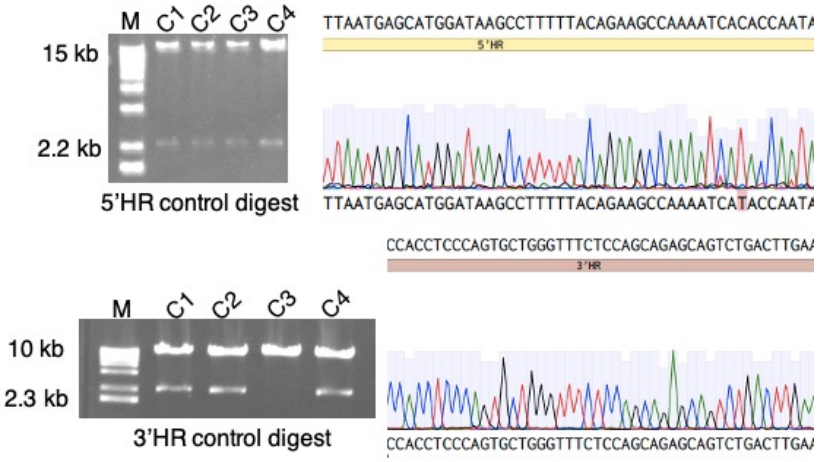

Figure 5.4.: **Cloning of the targeting construct**, Each cloning step was verified by control restriction digest, for which four clones were picked. For 5´HR, all clones showed band of the expected size and therefore, one clone was sequenced and aligned to the template. For 3´HR, three out of four clones exhibited the correct restriction digest, while sequencing of one of the clones showed an alignment to the template, M = marker, C = Clone.

5.1.2. Cloning of the sgRNAs for knock-out of exon 2

The sgRNA for ARKO was designed with the help of the online platform Benchling, available at www.benchling.com. Two suitable sgRNA sequences were chosen on the 5'and on the 3' side of exon 2, as shown in the Figure 5.5.

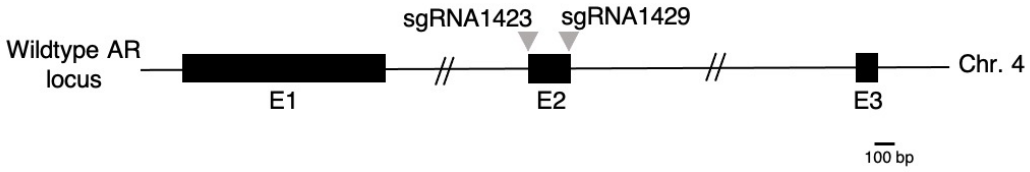

Figure 5.5.: **Location of sgRNAs for ARKO**, Two sgRNA were designed with the help of the online platform Benchling (www.benchling.com). sgRNAs were located on the 5'or 3'site of exon two.

Benchling was also able to analyze predicted efficiencies of the designed sgRNAs based on an on – and off – target score with maximum at 100. Predicted efficiencies are displayed in the table 5.14. sgRNA1423 shower an on – target score at 56 and sgRNA at 65. Off – target was for sgRNA1423 at 42 and for sgRNA1429 at 32.

sgRNA	On-target score	Off-target score
sgRNA1423	56	42
sgRNA1429	65	32

Table 5.1.: **Predicted efficiencies of sgRNAs**: Efficiencies of the sgRNA were based on on- and off- target score analyzed at the online platform Benchling (www.benchling.com).

Oligonucleotides for sgRNA were annealed and cloned into the empty backbone of the vector px330 (#34). After that, two clones per each sgRNA construct were picked and verified by restriction digest. As Figure 5.6 displays, only undigested plasmid was observed on the gel indicating a successful cloning procedure. One clone per each sgRNA construct was sequenced, which confirms the correct sgRNA sequence by annealing to the template. Constructs were inserted in the internal lab database and entitled as construct #125 for gRNA1929 and #114 for sgRNA1423.

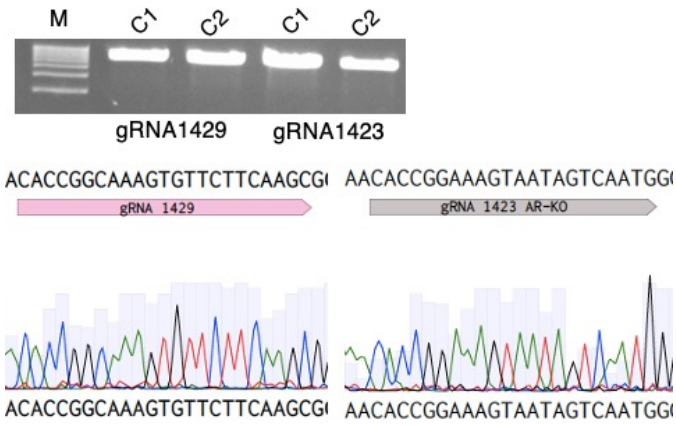

Figure 5.6.: **Restriction digest and sequencing of the sgRNA1423 and 1429**,sgRNAs were cloned into the backbone of the vector px330 (#34). Two picked cloned per each sgRNA showed only one band on the restriction digest indication successful cloning. One clone of each sgRNA was sequencing to verify the sgRNA sequence. Both sequencing results revealed the expected sequence after alignment to the template, C = clone, M = marker.

5.1.3. Establishment of androgen receptor knock-out in DT40 and PGCs

In our experiments, gene editing was based on a combination of homologous directed repair and CRISPR/Cas9 system. After the transfection with px330, a double-strand

break has occurred depending on the localization of sgRNA. The ARKO repair construct served as a template for homologous recombination. Due to presence of homologous arms, a selectable marker cassette was inserted instead of the exon 2, i.e., in between intron one and two. Therefore, the sequence of the exon 2 was completely knocked-out, as shown in the Figure 5.7. Both sgRNAs were tested separately together with the ARKO repair construct, and their functionality was determined.

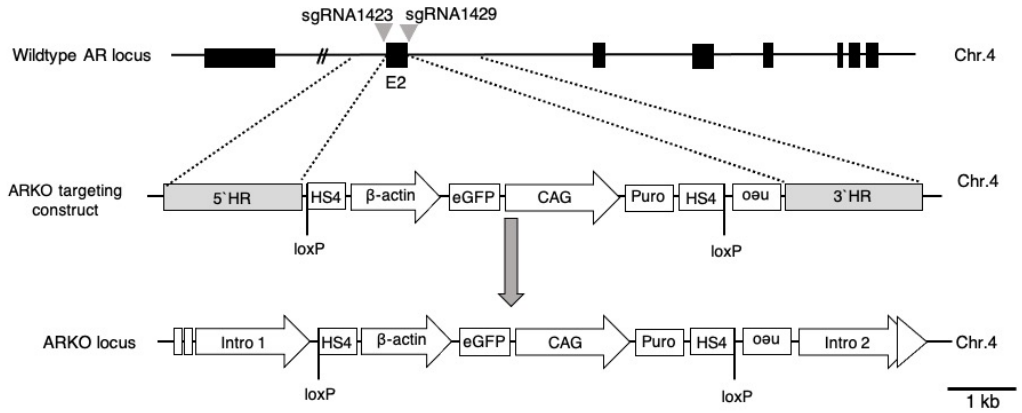

Figure 5.7.: **Homologous recombination and CRISPR/Cas9 system**, CRISPR/Cas9 system caused a double-strand break according to the specific localization of sgRNA in the exon 2 on its 3'or 5' end. Either sgRNA1423 or sgRNA1429 were used in combination with the ARKO repair construct. The ARKO repair construct containing homologous arms served as a template for repairing the double-strand break via homologous directed repair. This way, exon 2 was utterly knocked-out and replaced by a selectable marker cassette.

For the functionality determination, DT40 cells were chosen as a model cell line. DT40 were cultured under the standard conditions and subsequently electroporated with the sgRNA constructs and the ARKO repair construct. In total, three electroporations were performed - one reaction with 10 µg ARKO repair construct alone, and two other ones always in combination with 10 µg px-330-sgRNA1423 or 10 µg px-330-sgRNA1429 as shown in table 5.2 was observed. Electroporated cells were plated on 96-well plates, and 24 hours after transfection selected with puromycin for seven days. DT40s were frequently checked with a fluorescent microscope for eGFP to detect positive clones.

ARKO repair construct	px330-sgRNA1423	px330-sgRNA1423
10 µg	-	-
10 µg	10 µg	-
10 µg	-	10 µg

Table 5.2.: **Electroporation reaction mix**: In total, three types of transfections were performed in DT40. First, the ARKO repair construct alone was used. Second, ARKO repair construct and px330-sgRNA1423 was transfected. Third, the ARKO repair construct and px330-sgRNA1429 were transfected.

Positive clones were further expanded, and afterwards, gDNA was isolated and tested via PCR for the right integration of the selectable marker cassette. Only three eGFP positive clones were detected by electroporation with the ARKO repair construct alone but eight in combination with px330-sgRNA1423 and nine with px330-sgRNA1429 (table 5.3) was observed. Overall, all three types of electroporation showed positive clones (representative illustration is shown in Figure 5.8). Nevertheless, using CRISPR/Cas9 increased the editing efficiency about three times.

ARKO repair construct alone	ARKO repair construct + px330-sgRNA1423	ARKO repair construct + px330-sgRNA1429
3	8	9

Table 5.3.: **Number of positive clones detected in DT40 cells**: Only three clones were observed after transfection with the ARKO repair construct. Eight clones were detected after transfection with the ARKO repair construct + px-330-gRNA1423 and nine clones after transfection with the ARKO repair construct + px-330-sgRNA1429.

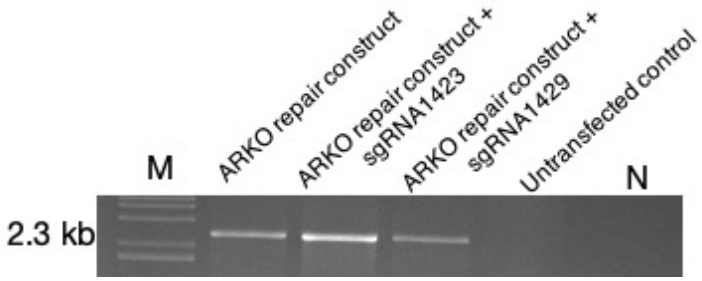

Figure 5.8.: **PCR for right integration on the 3' site**, Editing was successful in all three reactions, but in combination with CRISPR/Cas9, much higher efficiency was observed, N = negative control, M = marker.

Electroporation results in DT40 showed that both sgRNAs displayed similar editing efficiency. However, sgRNA1429 was located closer to the homologous arm, which is generally considered to be more efficient [128]. px330-sgRNA1429 was further used to generate ARKO PGCs.

5.1.3.1. Knock-out of androgen receptor in PGC LSL line 2-6

Gene editing in male PGC LSL 2-6 was performed to knock-out the AR exon 2 and generate ARKO clonal cell lines.

Male PGSs LSL 2-6 were electroporated with 10 µg of the ARKO repair construct (#116) and 10µg sgRNA1429 - px330 (#125). Five days post the electroporation, cells were selected with puromycin and regularly screened for clones expressing eGFP. In total, nine eGFP clones were detected and further expanded. From all clones gDNA was isolated and tested via PCR for the correct targeting. All clones were positive on 5' integration PCR. On 3'integration PCR, the clones C1-C4 and C6-C8 were positive. Clones, positive at both integration sites (in total seven) were further expanded, and 2-3 aliquots of $1x10^6$ cells were frozen down. C5 and C8 were negative on 3'integration PCR, and therefore, they were excluded from the following experiments.

ARKO repair construct	px330-sgRNA1429	Number of positive clones
10 µg	10 µg	7

Table 5.4.: **Transfection summary on PGC LSL 2-6 cell**: After transfection of line PGCs 2-6 was transfected with 10 µg of the ARKO repair construct and px330-sgRNA1429, seven clones with correct targeting was detected.

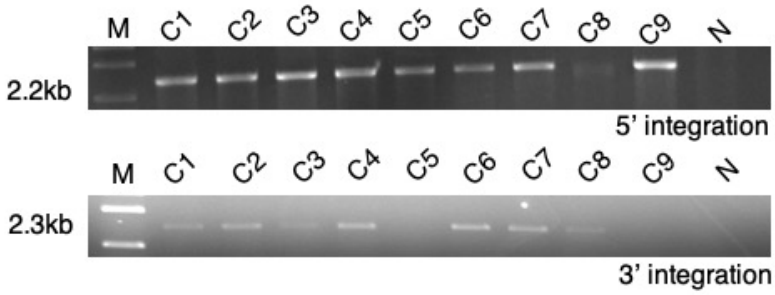

Figure 5.9.: **PCR for right integration on 3'and 5'site**, All tested clones were positive on 5'site, but only seven clones on 3'site. Clones C5 and C8 appeared negative on 3'site, and therefore, they were further excluded, M = marker, N = negative control, C = clone.

5.1.4. Generation of androgen receptor knock-out germline chimeras

On the day of injection, cells were spun and diluted in the concentration 3000 cell/µl. Always 1 µl, containing 3000 cells, was injected per embryo.

Injected cell lines	Injected eggs	Hatched chicks	Male chimeras
PGC LSL 2-6 ARKO Clone 2	56	30	12
PGC LSL 2-6 ARKO Clone 4	57	21	8

Table 5.5.: **Injection summary**: For clone 2, 56 eggs were injected providing 12 males chimeras. In case of clone 4, 57 eggs in total were in injected giving 8 male chimeras.

As shown in Table 5.5, 51 chickens hatched and 20 males from them were obtained. Female chicks were euthanized, and their gonads were dissected and examined for PGC colonization based on eGFP fluorescence. The colonization is displayed in the Figure 5.10.

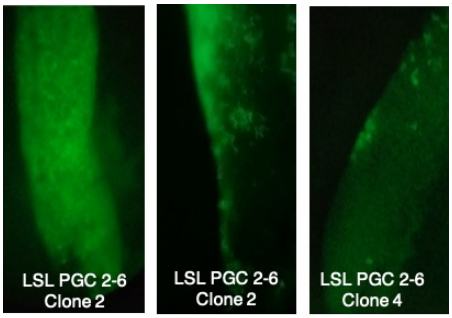

Figure 5.10.: **Colonisation of the gonad**, Female chicks were euthanized and their gonads were dissected. eGFP fluorescence confirms successful colonization.

5.1.5. Testing of germline chimeras with androgen receptor knock-out

Evaluation of the germline chimeras consisted of two main steps. Firstly, semen of the germline chimeras was collected and tested via PCR for the presence of the eGFP on a genomic level. Secondly, germline transmission was tested by breeding of the most promising chimeras based on the PCR results.

5.1.5.1. Semen analysis of germline chimeras with androgen receptor knock-out

Male chimeras were raised to sexual maturity, but earlier, starting from the 16th week of age, their semen was examined for the presence of the androgen receptor knock-out. Semen was collected two to three times a week, as described in chapter 4.4. From each sample, gDNA was isolated and tested via eGFP-PCR. Figure 5.11 shows a representative illustration of the PCR result. Positive eGFP bands can be observed by rooster nr. 42519, 42520, and 42677.

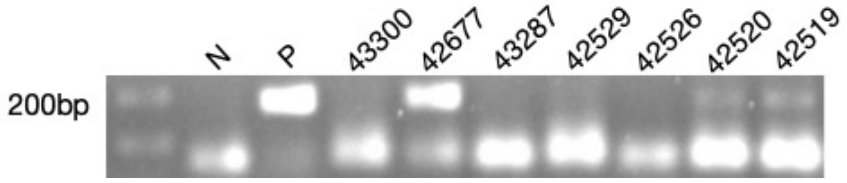

Figure 5.11.: **eGFP-PCR on semen samples from germline chimeras**, gDNA isolated from semen samples was tested for the presence of eGFP. Rooster number 42519, 42520 and 42677 showed a signal for eGFP, P = positive control, N =negative control.

From each chimera, four to five semen samples , in total, were collected. The rooster, having at least three times a positive band on eGFP-PCR, was considered a promising

candidate for further breeding. As shown in table 5.6, rooster 42677 was four times positive, and rooster 42526 was three times positive. Therefore, they were considered as good candidates and bred to the WT hens.

Rooster	Collection 1	Collection 2	Collection 3	Collection 4	Positive
42503	✓	-	✓	-	2
42511	-	-	-	-	0
42515	-	-	-	-	0
42516	✓	-	✓	-	2
42519	-	✓	-	-	1
42520	-	✓	-	✓	2
42523	-	-	✓	✓	2
42525	-	-	-	-	0
42526	✓	-	✓	✓	3
42529	-	-	-	-	0
43284	-	-	-	-	0
43287	✓	-	-	✓	2
43288	✓	-	-	-	1
43293	-	-	-	-	0
43296	-	-	-	-	0
42677	✓	✓	✓	✓	4
43288	-	-	-	✓	1

Table 5.6.: **Summary of the eGFP-PCR**:Collected sperm of germline chimeras was analyzed via PCR. The rooster with positive eGFP band on PCR is marked as ✓.

5.1.5.2. Testing of germline transmission of androgen receptor knock-out

The promising roosters number 42677 and 42526 were bred to WT hens, and eggs were incubated for seven days before checking for germline transmission. At ED7 embryos were examined for eGFP fluorescence. Once a positive embryo was found, eggs from the breeding were further incubated until hatching to obtain ARKO heterozygous offspring (ARKO $^{+/-}$). The results are summarized in table 5.7. From the breeding with rooster 42526, unfortunately, no positive embryo out of 181 cracked eggs was observed. In contrast to the breeding with rooster 42677, the first cracked egg was found eGFP positive (Figure 5.12).

PGC LSL 2-6 ARKO	Chimera	Screened embryos	Positive embryos
Clone 4	42677	1	1
Clone 4	42526	181	0

Table 5.7.: **Number of examined embryos**: Two chimeras were set in the breeding with WT hens. The first cracked embryo was found positive in breeding 42677. Unfortunately, no embryo out of 181 cracked eggs was found in breeding 42526.

Genomic DNA of the eGFP positive embryo was analyzed by PCR in comparison to gDNA from WT embryos. PCR was specifically designed to revealed, whether the selectable marker cassette was correctly inserted and exon 2 of AR was knocked-out. As seen in the Figure 5.12, the eGFP embryo was positive for targeting on 5', as well as 3' integration PCR. Surprisingly, no band was observed with positive control and, as expected, WT control was found negative.

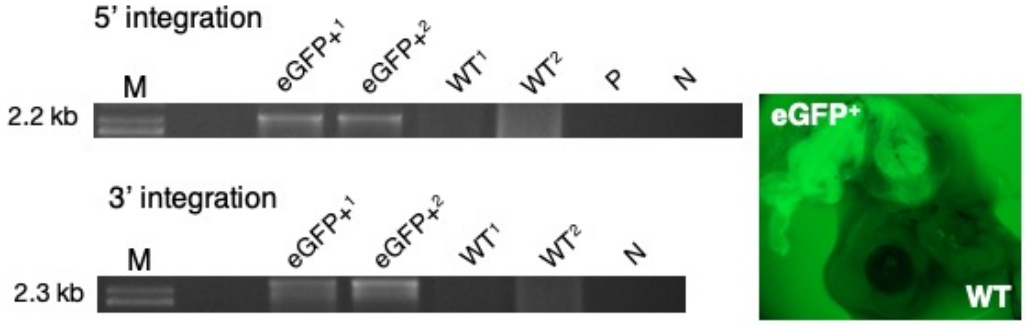

Figure 5.12.: **PCR for right integration of selectable marker cassette on eGFP positive and WT embryo**, two probes of eGFP positive and two probes of WT embryo were dissected at ED7. The eGFP positive embryo had correct integration of the selectable marker cassette on 5'and on the 3'site. And therefore, the correct targeting was confirmed. WT control was negative. P = Positive control, N = Negative control.

5.1.6. Hatching of the ARKO heterozygous and homozygous progeny

Since a positive embryo was found by the breeding of the rooster 42677 and WT hens, eggs of this breeding were further collected and incubated until hatch.

In total, eleven ARKO$^{+/-}$ chicken were obtained out of several hatches. At first, hatchlings were screened for eGFP expression, and from eGFP positive chickens blood was taken and tested via PCR for targeting of the androgen receptor. Figure 5.13 shows a representative illustration of PCR for right integration. Both hatched animals,

PGC LSL 2-6 ARKO	Chimera	Progeny	ARKO $^{+/-}$ progeny	Male	Female
Clone 4	42677	431	11	3	8

Table 5.8.: **Number of hatched progeny**: In total, 11 heterozygous ARKO progeny (three males and eight females) were hatched out of 431 chicks.

44576 and 44577, were positive for 5', as well as 3'site integration, and WT control was negative.

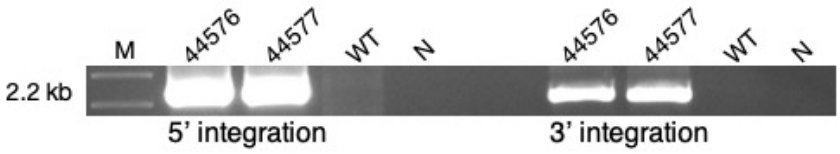

Figure 5.13.: **PCR for right integration of selectable marker cassette integration on first ARKO**, Chicks were sorted out according for eGFP after hatch. gDNA from eGFP positive chicks was isolated and tested via PCR for the right integration. 44576 and 44577 were positive for 5'and 3'integration, M = marker, N =negative control..

By breeding of the ARKO heterozygous male and female chickens, the ARKO homozygous offspring was obtained. At first, genotyping was established to distinguish between ARKO homozygous, heterozygous or wild type (WT). PCR was designed with specific primers. Upper band at 400 bp represents ARKO allele, while lower band at 300 bp represents WT allele. The primers were combined in one reaction. ARKO $^{-/-}$ displayed band only at 400 bp ARKO band, while ARKO$^{+/-}$ exhibited both of them. The representative illustration of the genotyping is displayed in Figure 5.14.

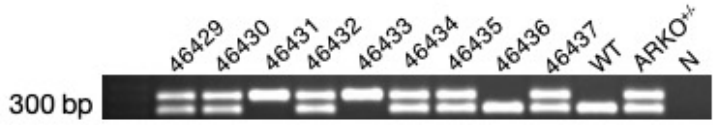

Figure 5.14.: **Genotyping of ARKO chickens**, To distinguish homozygous and heterozygous ARKO, genotyping PCR was established. Upper band (400bp) is specific for ARKO, lower band (300 bp) for wild type. ARKO$^{-/-}$ displayed only 400 bp ARKO band, while ARKO$^{+/-}$ exhibited both of them, WT = wild type, N = negative.

5.1.7. The phenotype of male and female ARKO chickens

During development and sexual maturation the phenotype of the ARKO animals was observed. ARKO heterozygous males and females did not exhibit any differences as compared to WT chickens. Normal fertility was observed in heterozygous males and females and therefore, breeding for ARKO homozygous was possible. During and after reaching sexual maturity, the impact of ARKO became evident in ARKO$^{-/-}$. In ARKO$^{-/-}$ males, sex-specific secondary sex characteristics such as comb, wattles and spurs were completely missing and they look liked juvenile chicks, except for the tail feathers that developed the normal male typical (sexually dimorphic) appearance (Fig. 5.15). Crowing and other sexual behaviors such as attempts to mount the females or any type of aggressive behavior was not observed in male ARKO$^{-/-}$, neither did they produce sperms. Only the tail feathers were longer than in females ARKO$^{-/-}$ which gave a possibility to distinguish between the sexes. Females also looked sexually indifferent and were similar to juvenile chicken; neither the female typical small comb nor the female-typical wattles were present in comparison to the WT females or ARKO$^{+/-}$. It is also worth mentioning that ARKO$^{-/-}$ females did not lay any eggs. The phenotype of the ARKO homozygous is displayed in the Figure 5.15.

Figure 5.15.: **ARKO homozygous and heterozygous chicken**, Lack of functional androgen/AR signaling strongly affected the phenotype of ARKO$^{-/-}$ chickens. ARKO$^{-/-}$ appeared to be sexually indifferent and both males and females looked like juveniles. Typical characteristics as wattles or spur were not present. These images were taken by Dr. Albertine Leitão at Max Planck Institute for Ornithology, Seewiesen.

5.1.8. Testosterone induced involution of bursa of Fabricius of the ARKO

In this experiment, the proof of the successful ARKO knock-out was performed. The bursa of Fabricius is known to degenerate if exposed to high levels of androgens during development [89, 90, 91]. Eggs from ARKO$^{+/-}$ breeding were dipped into testosterone solution and their bursae were dissected and analyzed by weight reduction and by histological staining.

5.1.8.1. Bursa to body weight ration analysis significantly decreased in WT embryos

In the WT group treated with testosterone, the embryo body weight was found in a range of 15.6-25.8 g and bursa weight 11.8-24.3 mg with a mean of the bursa to body weight ratio of 0.8609554 mg/g. The WT untreated group bursa weight was between19.9 and 25.4 g with bursa weights from 23.1 to 46.8 mg. The mean of the bursa to bodyweight ratio was 1.62686 mg/g.

In the homozygous ARKO group, which was treated with testosterone, the range of 15.4-23.4 g with bursa weight from 15.3 to 30.8 was measured, and the mean bursa to body weight ratio of 1.086054 mg/g was determined. The untreated embryos weighed between 18.7 and 26 g and their bursae between 12.1 and 43.9 mg. The mean of the bursa to bodyweight ratio was 1.43019 mg/g.

In the heterozygous ARKO group, treated with testosterone, embryo weights in 14.5-29.8 g were measured. The bursae's weight was between 15.3 and 28.1 mg, and the mean of the bursa to body weight ratio was 0.9451646 mg/g. In the untreated heterozygous group, the embryos' weight was from 13.5 to 25.4 g, and bursa weights 15.8-53.2 mg. The mean of the bursa to body weight ratio was 1.551571 mg/g. In the treated group significant difference was found between WT and homozygous ARKO group with a p-value of 0.01887. In the treated group, no significant difference occurred nor between WT and ARKO heterozygous (p=0.06295) neither between the ARKO heterozygous and the ARKO homozygous group (p=0.1128).

In the untreated group, no significant difference was found between WT and homozygous group (p=0.2503) and neither WT nor the heterozygous (p=0.5953). The homozygous and heterozygous untreated group also did not show any significant difference(p=0.2886). Bursae were further histologically analyzed, which is described in the following chapter 5.1.8.2.

In the WT group treated with testosterone, the embryo body weight was found in a range of 15.6-25.8 g and bursa weight 11.8-24.3 mg with a mean of the bursa to bodyweight ratio of 0.8609554 mg/g. The WT untreated group bursa weight was

between19.9 and 25.4 g with bursa weights from 23.1 to 46.8 mg. The mean of the bursa to bodyweight ratio was 1.62686 mg/g.

In the homozygous ARKO group, which was treated with testosterone, the range of 15.4-23.4 g with bursa weight from 15.3 to 30.8 was measured, and the mean bursa to body weight ratio of 1.086054 mg/g was determined. The untreated embryos weighed between 18.7 and 26 g and their bursae between 12.1 and 43.9 mg. The mean of the bursa to bodyweight ratio was 1.43019 mg/g.

In the heterozygous ARKO group, treated with testosterone, embryo weights in 14.5-29.8 g were measured. The bursae's weight was between 15.3 and 28.1 mg, and the mean of the bursa to body weight ratio was 0.9451646 mg/g. In the untreated heterozygous group, the embryos' weight was from 13.5 to 25.4 g, and bursa weights 15.8-53.2 mg. The mean of the bursa to body weight ratio was 1.551571 mg/g. In the treated group significant difference was found between WT and homozygous ARKO group with a p-value of 0.01887. In the treated group, no significant difference occurred nor between WT and ARKO heterozygous (p=0.06295) neither between ARKO heterozygous and ARKO homozygous group (p=0.1128).

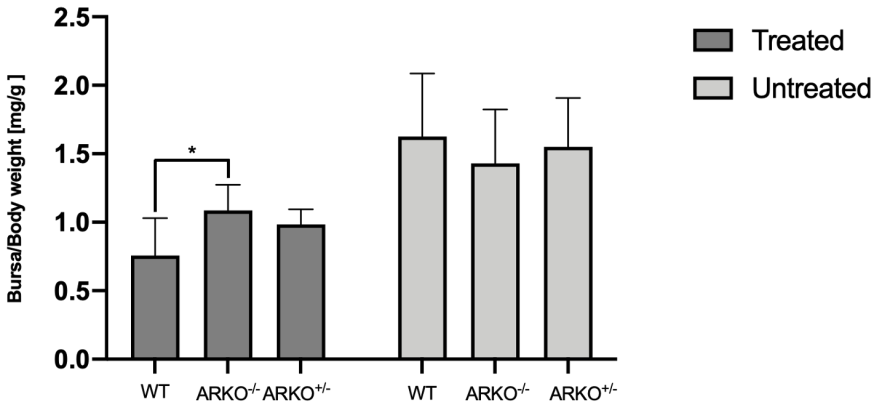

Figure 5.16.: **Bursa to body weight ratio of 18-day old embryos treated or not treated with testosterone**, At ED18, embryos were euthanized, and the bursa of Fabricius was dissected. The weight of the whole embryo and the weight of the bursa were tracked, and the bursa to bodyweight ratio was determined. WT, homozygous and heterozygous were analyzed via a non-parametric t-test with a significance level of 0.05. A significant difference was found only between WT and homozygous (p-value=0.01887) in the treated group. All other analyzed groups were not significant.

5.1.8.2. ARKO prevent from involution of bursa of Fabricius

Bursae were stained with Bu-1 for visualization of B-cells. Figure 5.17 shows the results of the staining. The untreated group displayed usual Bursa morphology with typical folds, in which B-cells were organized in follicles for WT and ARKO heterozygous and homozygous groups. In contrast, it is evident that for WT, the testosterone treatment had a powerful effect. B-cells were barely present in the bursa, and they were randomly distributed and did not form any follicles. The stroma of the bursa was not developed, resulting in the open centre of the organ. In the homozygous bursa, follicles were nicely formed and expressed structure and morphology similar to WT untreated bursae. Heterozygous bursae were partially affected by the treatment. B-cells were visible, and some of them were even organized in follicles. Nevertheless, the amount of the follicles seemed to be smaller compared to homozygous treated bursae.

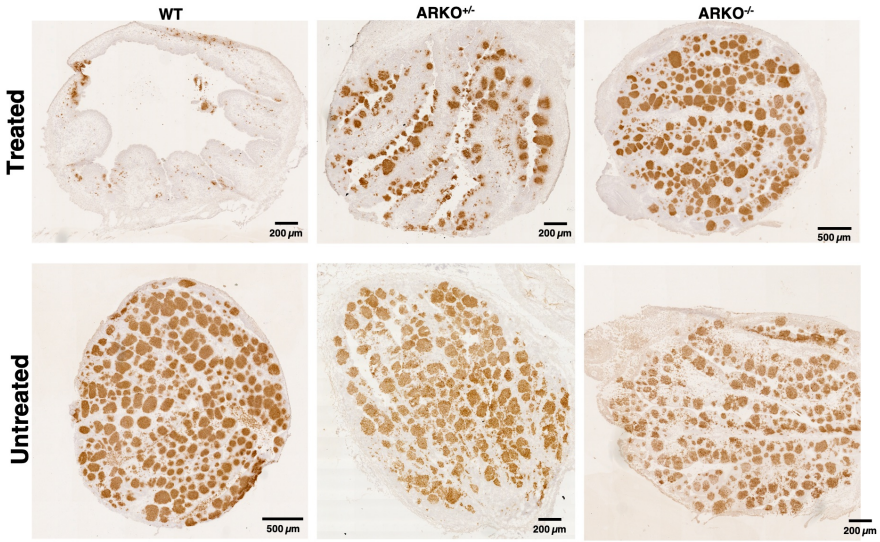

Figure 5.17.: **Histological analysis of the ARKO bursae treated and untreated with testosterone**, Bursae were dissected at ED18 and stained with Bu-1 for visualization of B-cells. The untreated group expressed the usual structure of bursa of Fabricius for WT, ARKO homozygous and heterozygous groups. In the bursa treated with testosterone, B-cells were barely present, and the morphology was strongly affected. The staining of the ARKO heterozygous bursa treated with testosterone showed the presence of the B-cells, and some of them were organized in follicles as well. Nevertheless, the amount of the follicles was reduced. The ARKO homozygous bursa seems to be not affected by the treatment at all and displays similar morphology as WT untreated bursa. One representative staining is shown (n>3/group).

5.1.9. Female ARKO chickens shows significant decrease in lymphocyte population after FACS analysis

At the age of 3, 6, and 12 weeks blood was taken and PMBCs (Peripheral blood mononuclear cells) the composition was analyzed by FACS. Figure 5.18 shows a representative illustration of the FACS staining. The cells were stained with viability dye (Alexa Flour 780) and then gated in FSC-H/FSC-A plot for determining single living cells. As an illustration, was shown staining of B-cells. For further quantification, the absolute number of immune cells (cell/µl) was normalized to 1000 living PBMCs according to the equation in Figure 5.18.

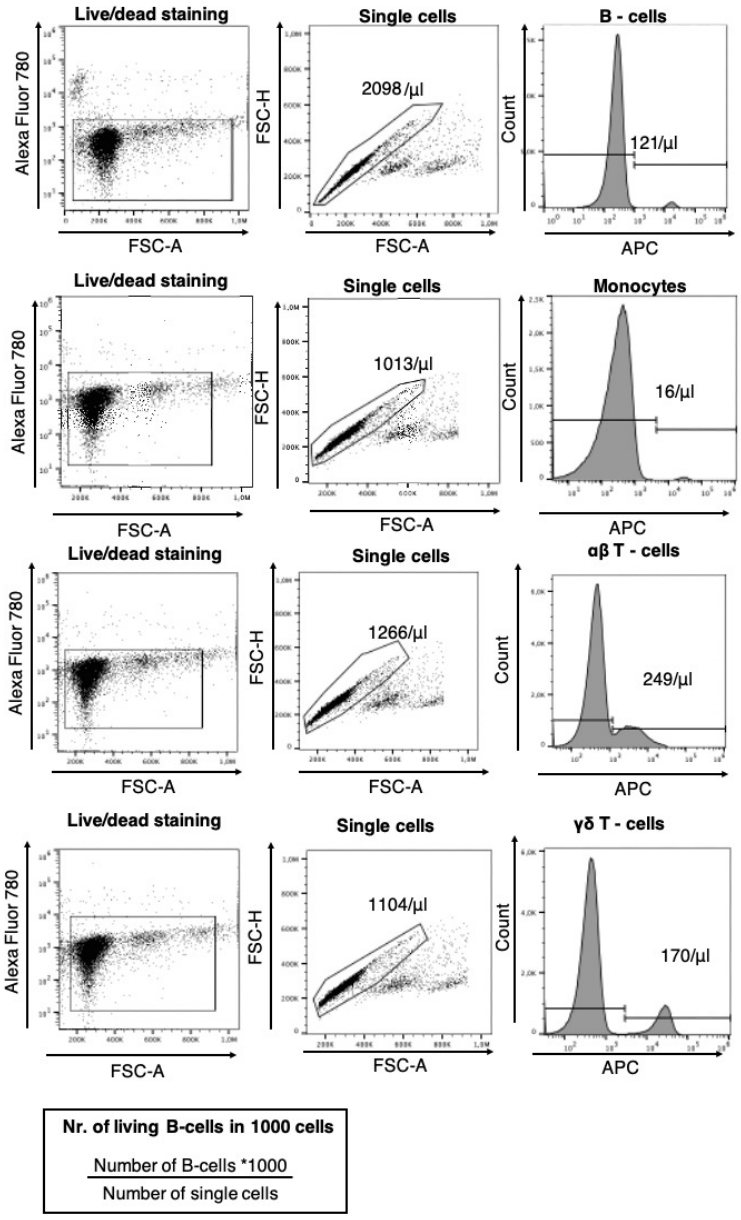

Figure 5.18.: **Representative illustration of the staining**, PBMCs were stained with viability dye to distinguish living cells and afterward gates with FSC-H and FSC-A for determining single cells. Finally, for each cell type, was determined a nr. of living single cells in 1000 cells according to the shown equation.

The lymphocyte analysis results are shown in the Figure 5.19 for males and females (Figure 5.20). The number of lymphocytes, namely B-cells, monocytes, αβ and γδ T-cells for homozygous and WT group, were analyzed at 3, 6, and 12 weeks of age. In males, while the number of monocytes was constant in WT, in homozygous ARKO chickens it was gradually decreasing. Although the slight difference between WT and the homozygous group was observed, it should be stressed out that it was not statistically significant. Statistical analysis was performed in R-studio via a non-parametric t-test with a level of significance of 0.05.

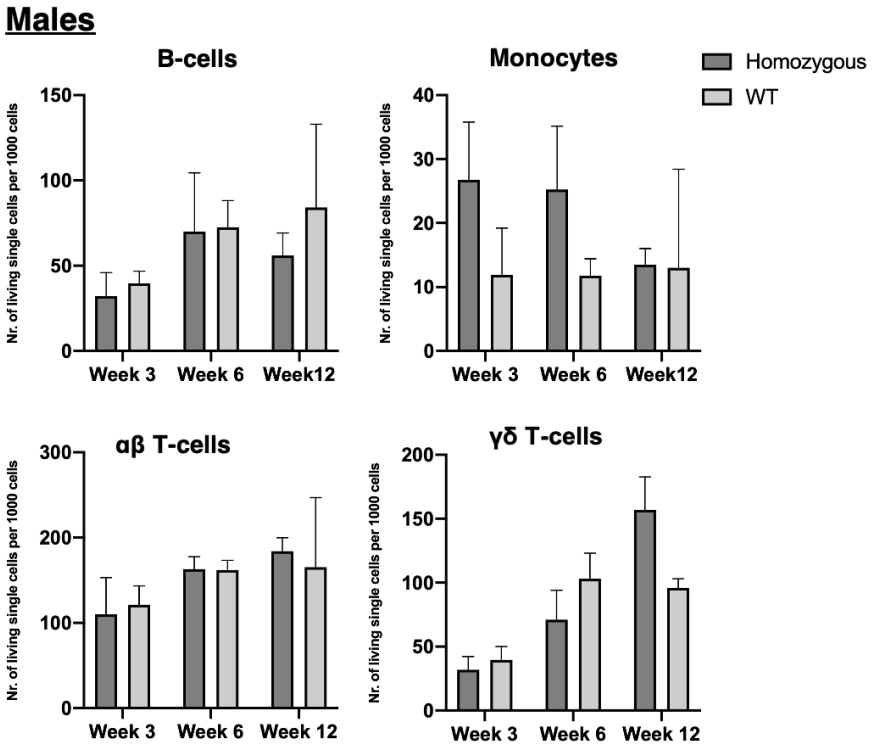

Figure 5.19.: **Lymphocytes analysis of the in male WT and ARKO chickens**, The lymphocyte population was measured at the age of 3, 6, and 12 weeks. B-cells, αβand γδT-cells were steadily rising over time for WT and for the homozygous group. While the population of monocytes displayed high values by the homozygous group for week 3 and week 6 and dropped at week 12, at the WT group, the values kept constant. No statistical significance was found after analysis with non-parametric t-test with a level of significance of 0.05, n≥3.

.

In females, the cell populations were steadily rising over the weeks in WT and ARKO$^{-/-}$ group. Only monocyte exhibited high value for week 3 and dropped at week

6 and 12. The statistics showed a significant difference between WT and ARKO$^{-/-}$ at several populations. Significant difference was found at B-cells population (p=0.03396, week 12). Monocytes exhibited significant difference for week 6 (p = 0.02456). αβT-cells of ARKO$^{-/-}$ were significantly decreased at week 12 (p = 0.04953) and γδT-cells were significantly decreased for week 6 (p = 0.02201) and 12 (p = 0.005123).

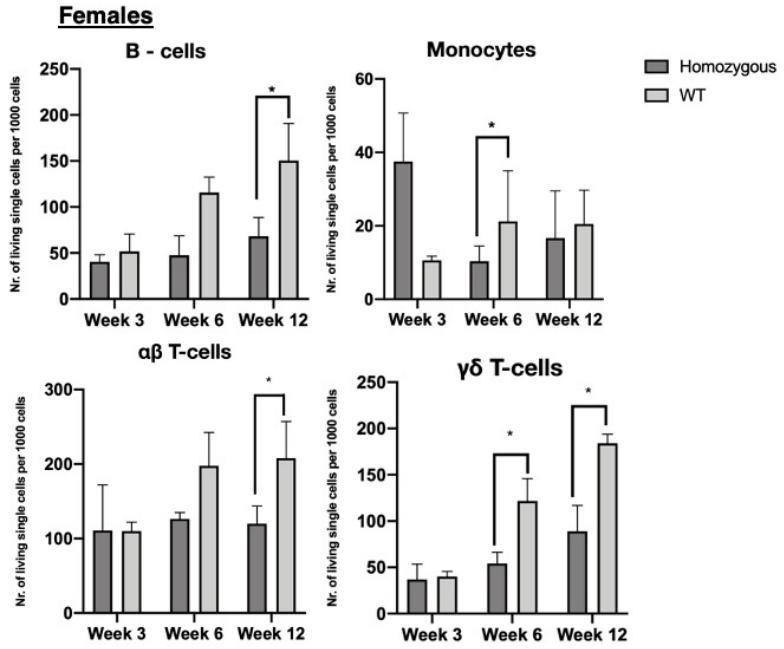

Figure 5.20.: **Lymphocyte analysis of female WT and ARKO$^{-/-}$ chickens**, The lymphocyte population was measured at the age of 3, 6, and 12 weeks. B-cells, αβand γδT-cells were steadily rising over time for WT and for the homozygous group. While the population of monocytes displayed high values by the homozygous group for week 3 and dropped at week 6 and 12, at WT group, the values kept constant. Significant difference was found at B-cells, monocytes,αβand γδT-cells. Statistical analysis was performed in R-studio via a non-parametric t-test with a level of significance of 0.05, n≥3.

.

5.2. Generation of ARKO Loop out chickens

In our ARKO homozygous chickens, generated in previous chapter 5.1, negative side effects such as body groWTh were observed. Therefore, ARKO PGCs were derived and

treated with the Cre recombinase in order to remove the selectable marker cassette and eliminate the side effect. ARKO loop out (LO) primordial germ cells were re-injected into chicken embryos to generate ARKO LO chickens. The scheme of the ARKO LO is shown in the Figure 5.21

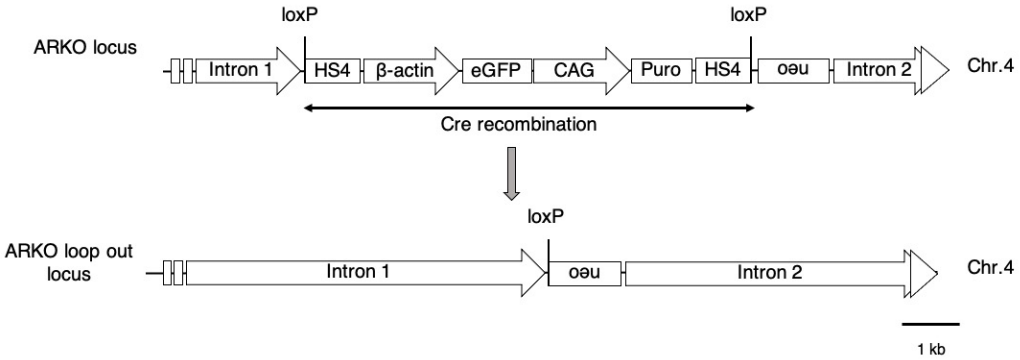

Figure 5.21.: **Scheme of the ARKO locus after transfection with Cre recombinase expression plasmid**, After the Cre construct transfection, the selectable marker cassette was excised. The remaining ARKO Loop out locus contains only one loxP site and neomycin in the reverse direction without promoter.

5.2.1. Derivation of ARKO PGCs from chicken embryos for ARKO loop out

Eggs from a breeding of the ARKO$^{+/-}$ animals were incubated for 65 hours. PGCs from these embryos were derived, and the rest of the embryo was kept for gDNA isolation for genotyping and sexing. Female lines were immediately sorted out, and male heterozygous and homozygous ARKO lines were further expanded.

Line	Genotype	Sex
1	Heterozygous	Male
2	Heterozygous	Female
4	Heterozygous	Female
5	Homozygous	Male
6	Homozygous	Female
8	Heterozygous	Female
9	Homozygous	Male
10	Homozygous	Male
11	WT	Male
12	Heterozygous	Male
13	Homozygous	Female
14	Homozygous	Male
15	Heterozygous	Male
16	Heterozygous	Male
17	Homozygous	Male
18	Heterozygous	Male
19	Homozygous	Male
20	Heterozygous	Female
21	Heterozygous	Female

Table 5.9.: **Genotype and sex of the ARKO PGCs** PGCs derived from ARKO breeding were genotyped and sex via PCR in order to distinguish ARKO and ARKO. Female ARKO were immediately sorted out and only male were kept.

Lines 1, 10, 12, 14, and 15 proliferated the most, and 5-6 aliquots of each line were frozen down. Lines 10 and 12 were chosen for further experiments.

5.2.2. Loop out of selectable marker cassette in ARKO PGCs

ARKO Line 10 (homozygous) and 12 (heterozygous) were expanded to reach five million cells, which was sufficient for one transfection. Each line was transfected with #105 Cre construct and kept under standard cultivation conditions, the eGFP fluorescence was decreasing. After seven days, PGCs were sorted by FACS and highly eGFP negative cells were sorted (FACS sorting was performed at the Department of Animal Sciences, LMU Munich, Prof. Dr. Bernd Kaspers). The gating is showed in the Figure Figure 5.22. In total, from five million cells of ARKO Line 12, 1.3 million eGFP negative cells and from seven million of ARKO Line 10 1.5 million eGFP negative cells were sorted.

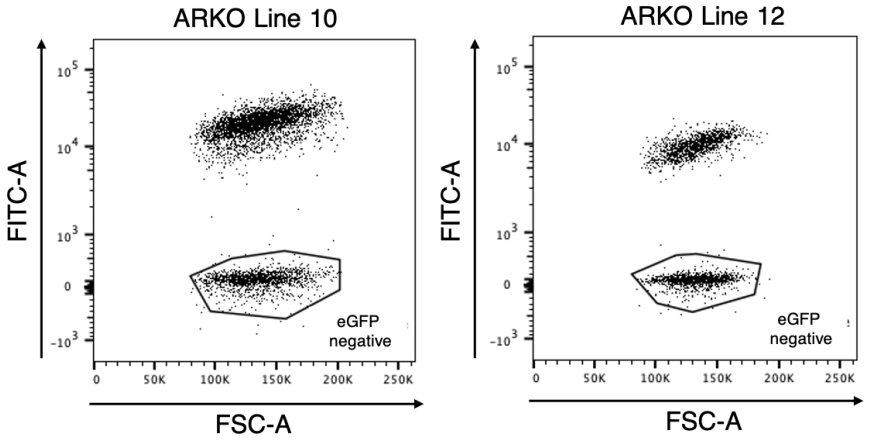

Figure 5.22.: **FACS Sorting of highly eGFP negative cells,** , Two ARKO Lines 10 and 12 were transfected with #105 Cre construct to loop out the selectable marker cassette with eGFP. PGCs were gated with FSC-A and FITC-A for sorting out these which were eGFP negative.

After the FACS sort, the ARKO LO primordial germ cells were analyzed via PCR. At first, a loop out PCR was performed and showed a band of the expected size at 1.4 kb (Figure 5.23) indicating a successful loop out of the selectable marker cassette. Secondly, Cre PCR was performed to examine the presence of the Cre gene in ARKO LO primordial germ cells. Genomic DNA was isolated 21 days post transfection with the #105 Cre construct. As Figure 5.23 indicates Cre was not detected.

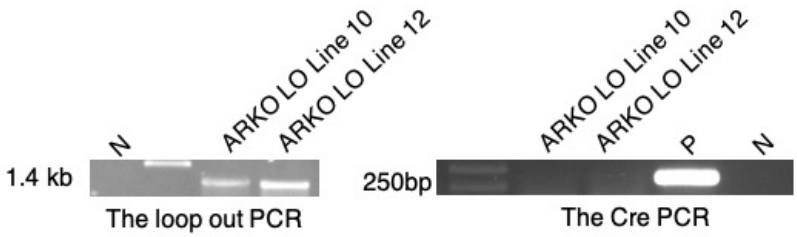

Figure 5.23.: **PCR analysis of the ARKO PGC after the FACS sort**, The loop out of the selectable marker cassette was proven via PCR for both transfected lines. 21 days after transfection with #105 Cre construct, PGCs were already negative for Cre

5.2.3. Generation of ARKO loop out germline chimeras

The ARKO LO line 10 and 12 were injected into LSL eggs in order to generate germline chimeras. PGCs were diluted concentration 3000 cells/µl and 1µl was applied on each embryo.

Injected cell lines	Injected eggs	Hatched chicks	Male chimeras
PGC ARKO LO Line 10	40	15	9
PGC ARKO LO Line 12	40	24	17

Table 5.10.: **Injection summary**: From each ARKO LO line, 40 eggs were injected. In case of ARKO LO Line 10, nine males chicks hatched. In case of ARKO LO Line 12, 17 male chicks hatched.

As shown in the table 5.10, 40 embryos of each line were injected and in total, nine males of ARKO LO Line 10 and 17 of Line 12 hatched.

5.2.4. Testing of the germline chimeras with ARKO loop

As described in chapter 5.1.5, the evaluation of the germline chimera consisted of two parts. Firstly, specific LO PCR on collected semen of germline chimeras and, secondly, testing of germline transmission.

5.2.4.1. Semen analysis of germline chimeras with ARKO loop out

The chimeras were raised to sexual maturity, and from the 16th of age, the semen was collected two to three times a week. The genomic DNA from semen was isolated, and the PCR specific for the LO locus was performed. Figure 5.24 shows a representative illustration of the PCR.

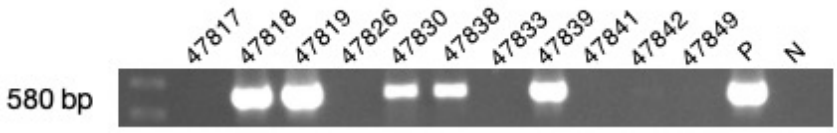

Figure 5.24.: **LO PCR on semen of the germline chimeras**, gDNA was isolated from the semen of the germline chimeras to examine the ARKO LO. The specific PCR was designed for this purpose. This gel figure showed that roosters 47818, 47819, 47830, 47838, and 47839 are positive, P = positive control, N = negative control.

In total, the semen samples were collected four times. In table 5.11 results of the LO PCR are shown. Chimera 47819 and 47830 were positive three times, chimera 47818 and 47839 twice. Chimera 47819 was selected for breeding to WT hens

Rooster	Collection 1	Collection 2	Collection 3	Collection 4	Positive
47814	-	-	-	-	0
47816	-	-	-	-	0
47817	-	-	-	-	0
47818	-	-	✓	✓	2
47819	✓	✓	✓	-	3
48722	-	-	-	-	0
47825	-	-	-	-	0
47826	-	-	-	-	0
47829	-	-	-	-	0
47830	-	✓	✓	✓	3
47832	-	-	-	-	0
47833	-	-	-	-	0
47838	-	-	✓	-	1
47839	-	✓	✓	-	2
47841	-	-	-	-	0
47842	-	-	-	-	0
47839	-	-	-	-	0

Table 5.11.: **Summary of the LO PCR**: Collected sperm of germline chimeras was analyzed via PCR. Rooster with positive LO band on PCR is marker as ✓.

5.2.4.2. Testing of germline transmission of ARKO loop out

The eggs from the breeding with 47819 were collected and incubated for seven days. At ED7, eggs were opened, and a piece of the tissue from each embryo was analyzed by PCR for germline transmission (Figure 5.25).

Figure 5.25.: **ARKO LO PCR on embryos**, 26 eggs were cracked at ED7, and from each embryo, a small piece of the tissue was taken. gDNA was isolated from each sample and specific LO PCR was performed to detect the ARKO LO. One out of 26 embryos was found positive, but only 12 probes are shown in the figure.

One positive embryo out of 26 was found (Table 5.15), and therefore all further eggs were kept until hatch to obtain ARKO$^{+/-}$ LO. Since the positive result was obtained already at chimera 47819, further breeding was not set up.

PGC ARKO LO	Chimera	Screened embryos	Positive embryos
Line 10	47819	26	1

Table 5.12.: **Number of examined embryos**: One chimera 47819 was set in the breeding with WT hens. One out of 26 embryos was found positive.

5.2.5. Hatching of the ARKO LO heterozygous progeny

The eggs from the 47819 breeding were further collected and incubated to obtain ARKO$^{+/-}$ LO. In two batches, five ARKO LO chickens out of 54 were hatched. The summary is displayed in the table 5.13.

PGC ARKO LO	Chimera	Progeny	ARKO $^{+/-}$ LO progeny	Male	Female
Line 10	47819	54	5	4	1

Table 5.13.: **Number of hatched progeny**: In total, 5 heterozygous ARKO LO progeny (four males and one female) were hatched out of 54 chicks.

Blood from the hatchlings was collected, and gDNA isolated from the blood was analyzed via LO PCR. Figure 5.26 shows a representative illustration of the PCR result, which displays that chicken 48567 was positive.

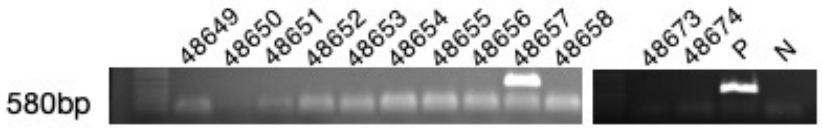

Figure 5.26.: **LO PCR on hatched offspring**, The blood from the hatchlings was collected, and gDNA was isolated. After LO PCR was performed, positive chicken 48657 was found.

The ARKO LO$^{+/-}$ chickens were bred to homozygosity and their phenotype was confirmed to be identical to ARKO$^{-/-}$ except the reduced weight gain and the enlarged crop.

5.3. Investigation of strategies for generation of reporter chicken line

This chapter describes different approaches of androgen receptor fusion to a tag in order to generate AR reporter chicken. AR fusion would allow to visualize AR without need on using any antibodies. Potential strategies for AR fusion are shown in the Figure 5.27.

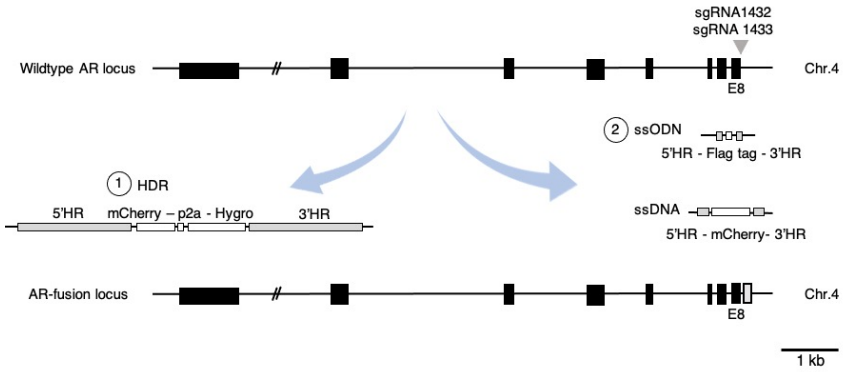

Figure 5.27.: **Strategies for androgen receptor knock-in**, To generated AR-fusion protein, two different strategies could be applied. Primarily, the homologous directed repair with a CRISPR/Cas9 system was tested. Secondly, PGCs were transfected with single-stranded oligonucleotide or single-stranded DNA and CRISPR/Cas9 system to generate AR fusion protein either with mCherry or with flag tag, HDR = Homologous directed repair, ssODN = single stranded oligonucleotide, ssDNA = single stranded DNA. Please note that displayed patters are only schematic and don't reflect the real sizes.

5.3.1. Cloning of the targeting vector for androgen receptor-mCherry fusion protein

Essentially, in our experiment a homologous directed repair together with CRISPR/Cas9 system was applied for the androgen receptor reporter chicken line. Similarly, as for ARKO, a targeting construct was generated. The targeting construct contained two homologous arms which surrounded a selectable marker cassette containing the mCherry and sequence for hygromycin resistance connected via self-cleaving protein p2a. The scheme of the targeting vector is displayed in the Figure 5.28.

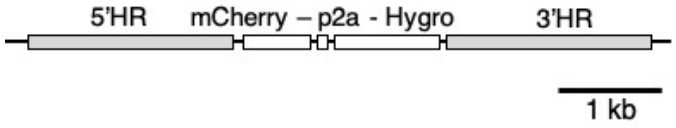

Figure 5.28.: **Targeting construct for AR fusion protein**, targeting construct was cloned with two homologous arm and selectable marker cassette congaing sequence for mCherry protein and gene for hygromycin resistance.

At first, a PCR for homologous arms and for selectable marker cassette was performed. Each PCR was run in four reactions, which were pooled and purified via gel extraction before Gibson Assembly. As Figure 5.29 displays PCR was successful and fragments of the expected size were amplified.

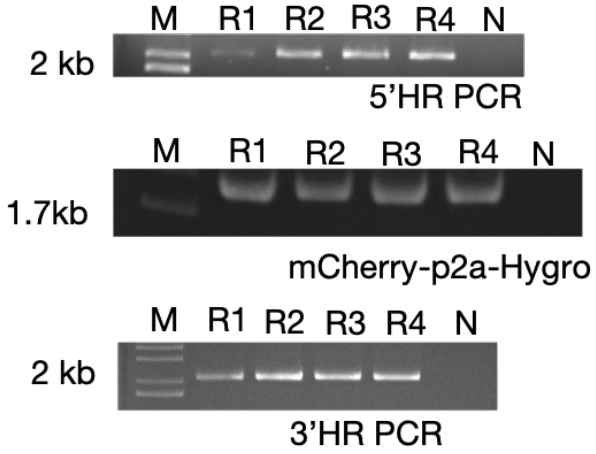

Figure 5.29.: **PCR for cloning of the targeting vector**, 5'HR, 3'HR and mCherry-p2a-hygro fragment were amplified via PCR. Each sample was run in four reactions, which were concentrated via gel purification of Gibson Assembly, M = marker, R = reaction, N = negative control.

At each cloning step, always four clones were picked and verified by restriction digest. At 5'HR and mCherry-p2a-hygro control digest all clones were correctly digested as displayed in the Figure 5.30. One clone was then sent for sequencing and aligned to the template. Figure 5.30 shows that all sequenced fragment were correct and aligned to the template sequence.

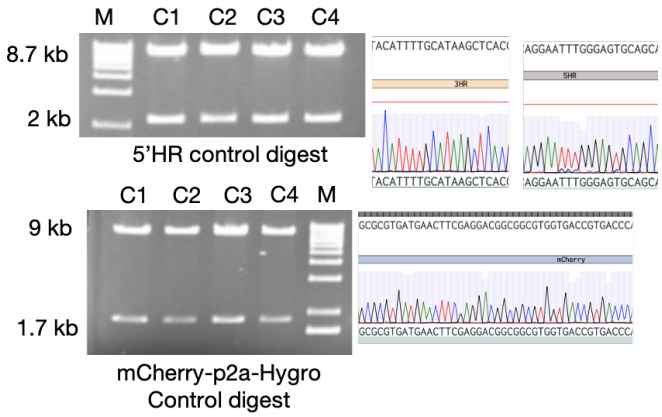

Figure 5.30.: **Restriction digest and sequencing of the targeting construct**,5´HR and mCherry-p2a-hygro fragments showed band of the expected size after restriction digest. Sequencing of all fragments successfully aligned to the template, M = marker, C = clone.

Targeting construct was entitled ARKI-mCherry-p2a-hygro and was entered into the database as #188.

5.3.2. Cloning of the sgRNAs for androgen receptor fusion protein

sgRNA for generation of androgen receptor fusion protein was designed with the help of the online platform Benchling, available at www.benchling.com. Two suitable sgRNA sequences were chosen, both close to the stop codon of the AR.

sgRNA	On-target score	Off-target score
sgRNA1432	65	46
sgRNA1433	63	46

Table 5.14.: **Predicted efficiencies of sgRNAs**: Efficiecies of the sgRNA were based on on- and off- target score analyzed at online platform Benchling (www.benchling.com).

Benchling was also able to analyse the predicted efficiencies of the designed sgRNAs based on an on – and off – target score with maximum at 100. Predicted efficiencies are displayed in the table 5.14. sgRNA1432 showed an on – target score at 65 and sgRNA1433 at 63. Off – target was for sgRNA1432 at 46 and for sgRNA1433 at 46.

Oligonucleotides for sgRNA were annealed and cloned into the empty backbone. sgRNA1433 was cloned into the backbone of px330, sgRNA into px330, px458 and px459. After that, two clones per each sgRNA construct were picked and verified

by restriction digest. For both clones and sgRNA only one band on the gel, was observed, which was a sign of successful cloning. One clone per each sgRNA construct was sequenced to confirm the correct sgRNA sequence. The result of the successful cloning and sequencing is displayed in the Figure Figure 5.31. Constructs were inserted in the internal lab database and entitled as construct #150 (px330-sgRN1432), #151 (px330-sgRN1433) and then #205 (px458-gRNA1432) and #206 (px4598-gRNA1432).

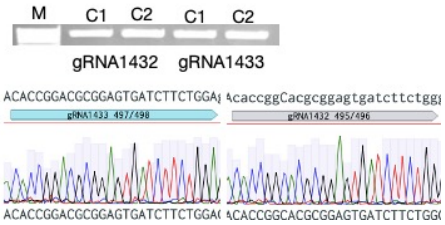

Figure 5.31.: **Cloning and sequencing of sgRNA1432 and 1433**,Oligonucleotides for gRNA were cloned into CRISPR/Cas9 vectors. On the gel successful cloning result is displays and sequencing was correctly aligned to the template, M = marker, C = clone.

5.3.3. Testing the sgRNA efficiencies with TIDE tool

After cloning of the sgRNA 1432 and 1433 their efficiencies were separately analyzed. Both sgRNAs were transfected into DT40 cells, which were kept in the culture for 48 hours. After that, the gDNA was isolated and the DNA fragment, where the expected double strand break should occurred, was amplified. The DNA fragment (Figure 5.32) was sequenced and analyzed via the TIDE tool (www.shinyapps.datacurators.nl/tide/).

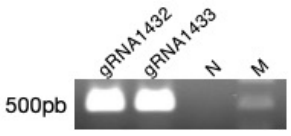

Figure 5.32.: **PCR for fragment amplification after CRIPSR/Cas9 transfection**, Fragments were the expected double strand break occurred were amplified and analyze via TIDE tool after sequencing

The TIDE tool compares the edited sequence to the WT sequence and provides the estimated insertion or deletion frequencies. The result from the TIDE tool (table 5.14) showed sgRNA1432 at 3.1 % and gRNA1433 at 2.1%. sgRNA1432 showed slightly better efficiency and therefore was used in further experiment.

gRNA	Efficiency
gRNA1432	3.3%
gRNA1433	2.1%

Table 5.15.: **sgRNA efficiencies estimated by TIDE tool**: gRNA1432 showed efficiency at 3.1 % and gRNA1433 at 2.1%. .

After the cloning, transfection in PGCs with targeting construct and px330-sgRNA1432 was performed several times. Unfortunately, this experiment was never successful and correct targeting was never observed. Therefore, in following chapter other possible approaches for AR fusion are described.

5.3.4. AR-flag tag fusion via ssODN transfection in PGCs

The PGCs were transfected with CRISPR/Cas9 construct px459-sg1432 and with ssODN containing short homology arms containing flag tag in the pilot experiment. After puromycin selection the genomic DNA was isolated and analyzed via amplicon sequencing. Although (Figure 5.33) the insertion of flag tag into the AR gene was successful, the editing efficiency was very low at 0.6 % . This strategy showed that an approach of ssODN is suitable for ARKI although with poor efficiency. To increase the editing efficiency, the transfection with px458-1432 followed by FACS sort was suggested. In this case, transfected cells express transiently GFP due to px458 and can then be easily discriminated. Following single cell screening could lead to finding of desired AR modification.

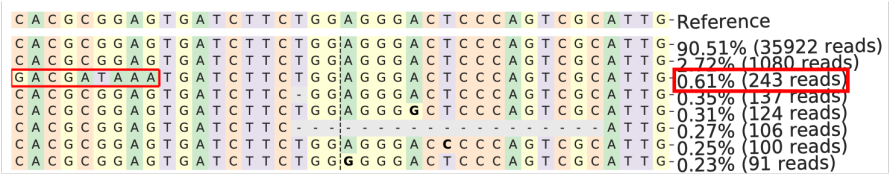

Figure 5.33.: **Amplicon sequencing of PGCs transfected with Flag tag ssODN**, PGCs were transfected with px459-gRNA1432 and selected with puromycin. The specific sequence was amplified via PCR and send for amplicon sequencing. The targeting with ssODN was positive but unfortunately with very low efficiency 0.6 %

5.3.5. Testing of AR signaling after tagging

To make sure that the tagging will not interfere with the AR signalling, AR reporter assay was designed to test the functionality of the AR after fusion. Firstly, four different

expression plasmids were created (Figure 5.34 A) by Gibson Assembly. Each plasmid expressed chicken AR alone or with a fusion of flag tag or mCherry. The chicken AR cDNA sequence was synthesized at IDT, Inc. and cloned in the expression vector pcDNA3.1. Subsequently, reporter plasmid pGL4.36[luc2P/MMTV/Hygro](Figure 5.34 A) was purchased from Promega, GmBh (Figure 5.34 A). pGL4.36 contained a sequence of Murine mammary tumor virus long terminal repeat (MMTV LTR) driving luciferase (Firefly) expression after activation of several nuclear receptors including an androgen receptor. Hek293T cells were transfected with the pGL4.36 alone or with an expression plasmids and stimulated with testosterone. Hek293T cells were then harvested and the cell lysate were analyzed in FLUOstar OMEGA Multidetection Reader.

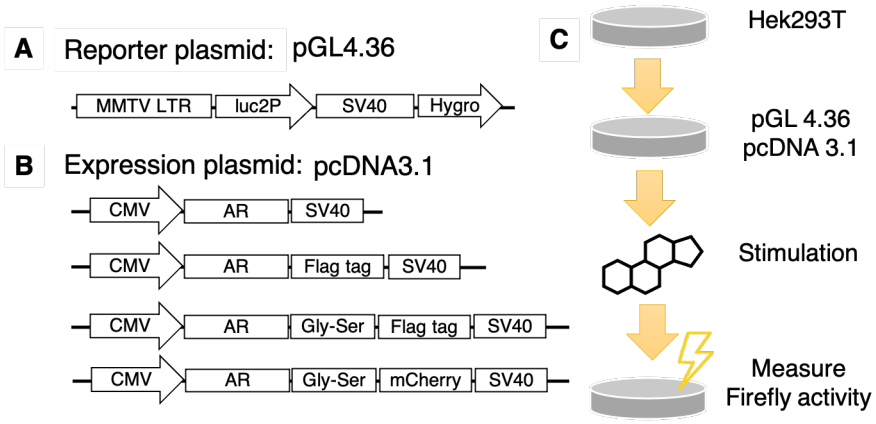

Figure 5.34.: **Experimental design of the AR reporter assay**, (A) For the purpose of the AR reporter assay, pGL 4.36 plasmid was purchased containing MMTV LTR driving the luciferase (Firefly) expression, (B) Furthermore, plasmids expressing chicken AR with or without different modifications were cloned, (C) The Hek293T cells transfected with pGL4.36 reporter and with plasmids expressing AR alone or in fusion with flag tag or witchery. After the stimulation with testosterone, the luciferase activity was measured from a cell lysate, adapted from [129]

Figure 5.35 demonstrates the ability of AR to bind the androgen response element like sequence (MMTV) by using different modifications of AR, namely AR-Flag tag and AR-mCherry. The modification in both cases involves Gly-Ser (GS) linker, but it should be noted that the flag tag fusion was also tested alone. Furthermore, three control groups were set up - untranslated cells, pGL4.36 only and plain AR. The results of the AR reporter assay showed a strong response after testosterone stimulation for flag tag and mCherry fusion. A stronger response was observed with GS linker indicating that it affects the AR signaling in a positive way.

Overall said, the tested modifications of the AR did not interfere with the AR signaling.

Moreover, both, the AR-mCherry and AR-flag tag fusion expressed a solid signal after testosterone stimulation and could be used for further experiments.

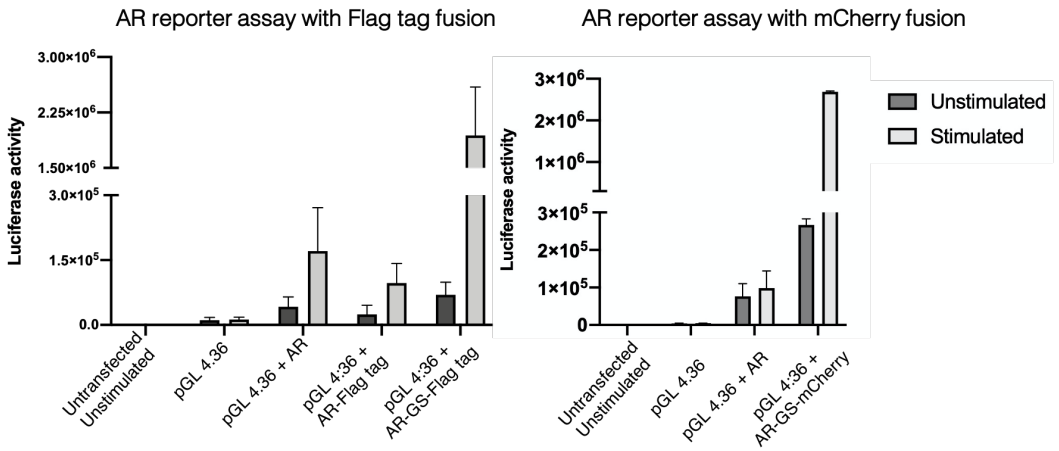

Figure 5.35.: **AR reporter assay**, Hek293T cells were transfected with reporter plasmid pGL4.346 based on luciferase expression and expression plasmids containing different AR modifications. The strongest signal was detected after stimulation with AR-GS-flag tag and AR-mCherry. These results indicate that fusion of the AR with flag tag of with mCherry does not interfere with the AR signaling.

5.3.6. Localization of AR-mCherry fusion protein via confocal microscopy

After the functional test of AR-mCherry fusion protein, it was necessary to analyze the protein translocation to the nucleus. For this purpose, Hek293T cells were transfected with pcDNA 3.1 AR-mCherry expression construct. Cells were stimulated with testosterone for 24 hours, fixed with 4 % PFA and stained with DAPI. The microscopy was performed at Chair Plant System Biology of Prof. Claus Schwechheimer at TUM with technical support from Dr. Philipp Denninger.

Figure 5.36 shows a representative illustration of the microscopic analysis. Several observations showed that a AR-mChery complex under the unstimulated conditions was localized mainly in the cytoplasm and the overlay with the nucleus was not significant. However, it was clear that the AR-mCherry complex translocated to the nucleus after stimulation with testosterone as shown in overlay images.

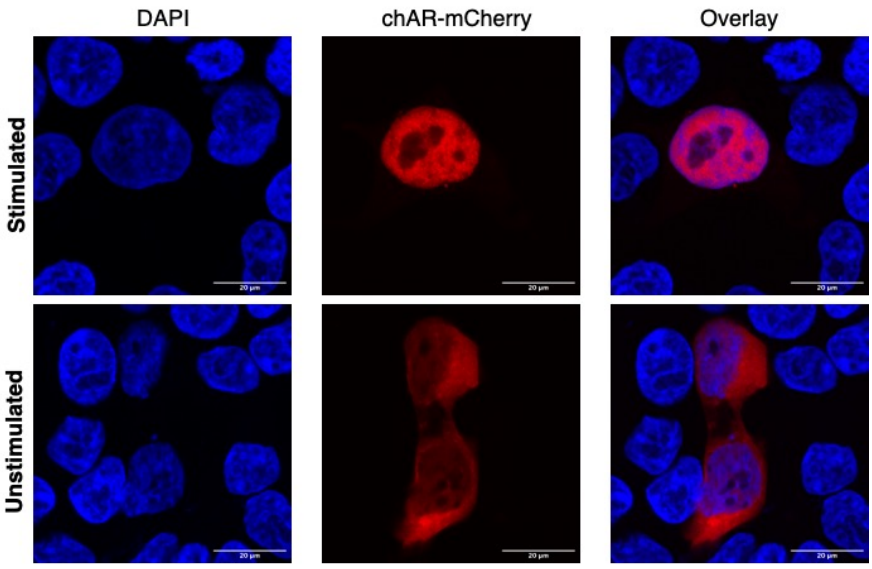

Figure 5.36.: **Localization of the AR-mCherry complex**, Hek293T cells were transfected with pcDNA3.1-AR-mCherry, stimulated with testosterone and counterstained with DAPI for nuclei visualization. For the unstimulated sample, localization mainly in the cytoplasm was observed. Stimulation with testosterone caused a strong translocated of the AR-mCherry to the nucleus. One representative sample of n>3 is shown.

It was evident that the AR-mCherry complex can translocate to the nucleus. Furthermore, from the digitized images, fluorescent intensity was evaluated with Fiji (Image J) and statistically analyzed in R-studio. As Figure 5.37A shows, at each image, the fluorescent intensity was measured in cell nucleus and in the cytoplasm. The intensity ratio (Figure 5.37C) was calculated and a non-parametric t-test with level of significance $p<0.05$ was performed. A significant difference was found between the treated and untreated group (Figure 5.37 B) with p-value 2.855×10^{-8}.

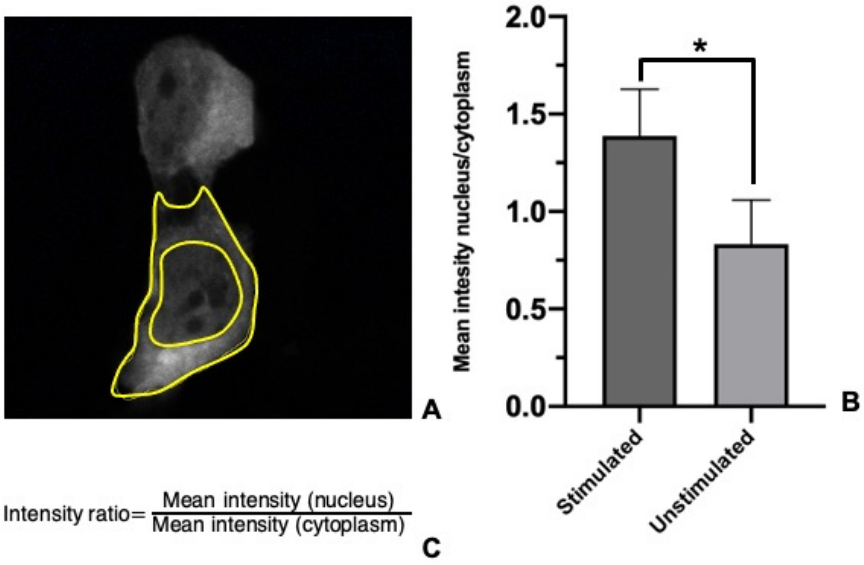

Figure 5.37.: **Fluorescent intensity ratio analysis**, Microscopic images were analyzed with Fiji (Image J) (A) and the intensity ratio was calculated with shown equation (C), The intensity ratio of stimulated and unstimulated was compared with non-parametric t-test with level of significance 0.05 ($\geq$10). The intensity of the stimulated group was significantly increased with p-value of 2.855×10^{-8}.

6. Discussion

In mammals, sex hormones play a major role in sexual development and the development of secondary sexual characteristics. The hormone regulation dominantly determines the male or female phenotype depending on gonad sex determination [17, 18].

In contrast, in chickens, it was proposed that cells possess so-called cell autonomous sex identity (CASI). In this concept, each cell obtains somatic identity to develop independently from the hormonal environment . Therefore, sexual development might be possible without effect of androgens or estrogens [5, 130].

Still the effect of androgens can't be overlooked and it is well known that androgens play an important role in avian physiology or behavior. It is evident that secondary sexual characteristics in males such as the large comb, the wattles or spurs are clearly androgen dependent [56]. Furthermore, similarly as in human or mice, testosterone influence the fat metabolism. Caponization (castration) of male chickens increased the fat accumulation in abdominal or subcutaneous areas [59]. Additionally, vocalizations such as crowing behavior is dependent on testosterone levels and can be induced by testosterone administration in female Japanese quails [62]. Moreover, the role of androgens in brain development and song related behaviors in song birds is irreplaceable [131, 80].

These studies are only a small part of the broad range of androgen sensitive effects known for avian species. In my thesis, the ARKO chicken was generated to study the effect of AR/androgen signaling for the chicken sexual development and for the development of secondary sex characteristics including the immune system.

6.1. Generation of ARKO chicken

The gene of AR is located on the somatic chromosome 4 in chicken [132]. Eight exons code for a protein consisting of three functional domains [30]. First, the ligand-binding domain (LBD) is important for hormone binding, which leads to dissociation from the heat shock proteins and conformational changes switching the receptor to transcriptional factor [133]. Second, the N-terminal domain (NTD), responsible for

transactivation, acts as a coactivator for binding testosterone or dihydrotestosterone [30]. Third, the DNA-binding domain (DBD), an essential part of the protein, interacts with the androgen response element (ARE) in DNA. DBD is encoded by both exon 2 and exon 3 [7]. This indicates that both exons became suitable for the knock-out since the DBD is fundamental in the signaling process after testosterone stimulation [32, 33, 34].

This fact can be supported by the naturally occurring androgen insensitivity syndrome (also called syndrome of testicular feminization - tfm) described in rats, which was first analyzed in 1991 on a genetic level. He *et al.* revealed point mutation and frameshift mutations causing a premature stop codon leading to missing DNA- and ligand-binding domain. Phenotypes characterized by strong feminization, resistance to testosterone, and infertility show the importance of both DNA- and ligand-binding domain [6].

It should be mentioned that the DNA-binding domain comprises two zinc fingers. Interestingly, exon 2 codes the first zinc finger containing three amino acidic residues, glycine, serin, and valine, that directly interact with the ARE. This suggests that it has a significant impact on AR functionality [84]. Since the DBD is the most conserved part of the AR among all species, we assumed that an androgen insensitivity syndrome could also be induced in chicken [30].

In mice, three studies generated a knock-out of exon 2 of AR [9, 39, 41]. Yeh *et al.* and De Gant *et al.* reported that knock-out of exon 2 of the AR resulted in male mice with feminized external genitalia, reduced testes size, and poorly developed scrotum. Testes were present in a low abdominal area similar to the phenotype of mice with a syndrome of testicular feminization. Histological analysis of the testes showed less cellular morphology with very thin seminiferous tubules compared to WT mice [9, 39]. Female ARKO mice did not show an external phenotype, but they suffer from subfertility with lower oocyte production and decreased levels of progesterone [41].

Based on the knowledge from tfm, ARKO mice models, and AR receptor structure, we proceeded with the knock-out of exon 2.

Prior to our experiment, one of the open questions was the ability of ARKO PGCs to migrate into the gonad and differentiate in functional sperms. Although several publications discussed AR expression in germ cells in mice, it remained unclear whether the expression is necessary for spermatogenesis [134, 135]. The answer to that was reported by Tsai *et al.*, where the exon 2 of AR was a knock-out in germ cells. The phenotype of germ cell knock-out mice was normal and did not differ from WT males. Serum testosterone levels were within the normal range, and spermatogenesis gave rise to functional sperms [47]. These results show that in chicken, normal spermatogenesis and development of functional sperms can be expected. Furthermore, spermatogenesis is a process highly conserved throughout vertebrates, indicating that the impact of

AR/androgen system in chicken germ cells differentiation might not be marginal [136].

Our experiments confirmed that functional AR in germ cells is unnecessary for spermatogenesis. Otherwise, an alternative approach had to be applied. For example, the strategy described for ARKO mice models, in which the Cre/loxP system is used [9, 39, 41]. Here, two chicken lines are generated. The first one, AR-loxP chicken, would be established by gene targeting in PGCs to insert the loxP sites upstream and downstream exon 2 in the intron region. Here the splicing of the AR should not be altered. The second one, ARKO chicken, would be created by breeding AR-loxP chicken and Cre expressing chicken.

Another option could be an inducible knock-out to switch off the androgen receptor at specific time points during the development. In this case, Cre expression would be induced by the administration of certain chemicals. As an example, tamoxifen is used for inducible knock-outs in mice [137]. The question is, of course, whether it also applies for chicken since tamoxifen is an antagonist of estrogen receptor and its administration affects the concentration of steroid hormone and influences the egg-laying [138]. Therefore, the phenotype of androgen receptor knock-out could be compromised by inhibition of estrogen receptor.

6.2. Strategies for the generation of an androgen receptor reporter chicken line

In mice and humans, AR is usually stained with anti-androgen receptor antibodies. Unfortunately, no reliable antibodies for chicken androgen receptors are available. Tagging with a fluorescent protein would allow studying the expression of AR *in vivo* as well as *in vitro* (5.3).

Introducing foreign sequence into a specific genomic locus is widely used and brings a great advantage to endogenous label proteins, introducing SNPs or generating humanized animals for studying disorders as Huntington [139] or Alzheimer's disease [140]. Cre expressing mice are also a powerful tool for conditional knock-outs via Cre/loxP system [141]. Furthermore, fluorescent animals possess an advantage providing system for cell imaging, cell tracking, or treatment *in vivo*. Mice models were mainly generated by inserting GFP, mCherry, or dTomato in ROSA26 because no lethal effect in this safe-harbor integration side was found [142].

During the generation of an androgen receptor reporter chicken line, we have hypothesized that fusion proteins might affect the function of the receptor. It was taken into consideration that tagging of AR might interfere with the signaling. To double-check

an influx of protein tags on the functionality of the chicken AR, a reporter assay for chicken AR was designed. In this assay, plasmids expressing different AR receptor modifications were generated and quantified using luciferase reporter plasmid, which was commercially purchased. Luciferase was expressed under murine mammary tumor virus long terminal repeat (MMTV LTR). It has been proposed that the MMTV LTR sequence acts like an androgen response element (ARE) [143]. As already mentioned, this is the essential part of the signaling, where AR binds in the nucleus to ARE, leading to a direct change in the gene expression. The binding of the testosterone stimulated AR to the MMTV LTR induced luciferase expression, and its intensity was afterwards measured. Identification and amplification of the chicken ARE might be very challenging since it has not been validated yet. Moreover, the process of ARE verification would include several additional assays, which is demanding with uncertain results. Therefore, MMTV LTR served as a good tool.

Reporter assays are commonly used in human medicine to test the activity of AR during prostate cancer progression [144], binding efficiency of different steroid ligands [145, 146] or endocrine disruptors blocking the androgen activity [147]. Our assay confirmed that AR either fused with flag tag or with mCherry transfers the signal and did not suffer from impaired signaling. Interestingly, AR fused with flag tag or with mCherry exhibited a higher luciferase signal (with Gly-Ser linker) compared to unmodified AR. In contrast, a fusion of AR with GFP slightly decreased the binding affinity, although the difference between AR and AR-GFP was not statistically significant [148]. However, some studies suggested that fusion with fluorescent proteins or other peptides can extend the half-live of proteins, which might prolong the presence of the fusion protein in the cytoplasm and reflect higher luciferase expression [149]. Furthermore, linkers are designed to improve the biological activity and to increase the expression yield, which might also contribute to the increase.

After verification that AR-mCherry fusion protein is fully functional, we confirmed by confocal microscopy that after stimulation AR-mCherry translocates to the nucleus. It was not particularly surprising since several studies reported a successful translocation with AR-GFP in mammals [148, 150, 151].

For a generation of an androgen receptor reporter chicken line, at first, homologous directed repair with CRISPR/Cas9 was applied. Primordial germ cells were transfected with a targeting construct containing homologous arms, the sequence coding for mCherry protein, and a gene for hygromycin resistance. It was expected that mCherry inserts in-frame 3' before the AR stop codon and that PGCs develop resistance to hygromycin. No additional promoter was added. Therefore, mCherry protein and a gene for hygromycin resistance should be expressed under the promoter of the androgen receptor.

The transfection of PGCs with targeting construct for a fusion of the mCherry to AR was

performed several times, but no positive editing was detected. In this case, PGCs have never been able to survive antibiotic selection. One of the possible explanations could be that expression of AR in PGCs is not strong enough to resist antibiotic selection. As was already mentioned in chapter 6.1, the expression of AR in germ cells in mice was examined but without a clear outcome. Nevertheless, mice model with specific knock-out of AR receptor in germ cell and our ARKO chicken model showed that AR is not necessary for sperm development [47]. Moreover, in our experiments, the AR expression in PGCs was tested on mRNA level. The RT-PCR of AR revealed a weak band on gel electrophoresis. Therefore, AR tagging and selection with antibiotics might be challenging. Even if positive targeting would occur, PGCs might not be able to survive the antibiotic selection.

To overcome the drawback of a low level of AR expression, we have suggested approaching the transfection of PGCs with single-stranded oligonucleotides (ssODN) and CRISPR/Cas9. Gene editing using single-stranded oligonucleotides is a standard tool for introducing single base pairs substitutions or point mutations with high efficiency [152, 153]. Nevertheless, it has also been shown that ssODN can be used for bigger insertion like loxP sites [154] or even GFP reporter cassette [155].

Our ssODN contained a flag tag for AR labeling and short homologous arms. After analysis by amplicon sequencing, a positive in-frame insertion of flag tag was observed, although with very low efficiency.

In order to increase the efficiency of gene editing, in the future, CRISPR/Cas9 construct expressing transiently eGFP will be used. This way, transfected PGCs can be enriched by FACS sort. Sorted cells have to be seeded as single-cell clones and screened for in-frame AR tagging. This might be more time-consuming but more efficient. On the other hand, it helps to overcome the issue with AR expression levels in PCGs.

As an alternative, CRISPaint (CRISPR-assisted insertions tagging) technology could be also considered. CRISPaint is based on CRISPR/Cas9 causing a double-strand break at the desired location, while at the same time the tag is provided in the donor plasmid and inserted via non-homologous-end-joining. This method provided an efficient in-frame tagging even without antibiotic selection [156]. For our experiment, this could higher the efficiency of gene editing although an enrichment by FACS sorting would be necessary.

Unfortunately, due to time restrictions, I could not establish the AR reporter chicken line.

6.3. ARKO affects the sex secondary dimorphism

Until the generation of ARKO heterozygous animals (ARKO$^{+/-}$), it was not entirely clear whether the knock-out of only one allele results already in a specific phenotype. In mice and humans, AR is present on the X chromosome as a single copy in males and two copies in females. In case that natural mutation in males occurs, the phenotype is directly evident and leads to hemizygosity. Hence, in males, the heterozygous phenotype can't be described. Therefore, the generation of female ARKO mice was for a long time almost impossible as male individuals were completely infertile. However, the Cre/loxP system also allowed the production of female ARKO mice [4]. In chicken, ARKO$^{+/-}$ exhibited a normal phenotype with no difference in comparison to WT chicken in males as well as in females. It might be due to AR present in two copies in chicken, and just one copy seems to be sufficient for sex development and fertility. Alternatively, AR expression of the single allele might be upregulated in order to compensate for the lack of the second AR allele.

Subsequent breeding gave rise to homozygous ARKO chicken (ARKO$^{-/-}$). During and after reaching sexual maturity, the impact of dysfunctional AR/androgen signaling became evident in males as well as in females. ARKO$^{-/-}$ roosters did neither exhibit secondary sexual phenotypes (except the tail feathers) nor sexual behaviors and did not produce any sperms. My findings correlate with data published in mice knock-out models or human androgen insensitivity syndrome [4, 133]. Nevertheless, it should be stressed that nonfunctional AR/androgen signaling in male mice and in men led exclusively to a female phenotype. For instance, human patients with complete androgen insensitivity syndrome (CAIS) had an entirely female appearance and female external phenotype, which could not be distinguished from a healthy woman without deeper examination [157, 158]. Simply speaking, based on knowledge from humans and mice, one could expect to generate a rooster with a phenotype of a hen. As shown in (Figure 5.15) this was not the case for chickens, since roosters looked more like juvenile chicks rather than like hens. Similarly, ARKO$^{-/-}$ hens did not develop any sex dimorphism and at first sight, were sexually indifferent. They did not lay any eggs suffered from infertility like ARKO$^{-/-}$ roosters since no eggs were present in the ovary.

ARKO female mice exhibited decreased fertility with defective follicle maturation. Nevertheless, external phenotype remained unchained [4]. Surprisingly, and different to mammals, in ARKO$^{-/-}$ hens, the phenotype was visible at first sight, and the nonfunctional AR/androgen system had a severe influence on the development of secondary sex characteristics as well as on fertility. These findings suggest that the sex development of female birds seems to be much more androgen-dependent than in female mammals.

It has to be pointed out, that estrogens can contribute to the sexual development and might modify sexual behaviours of birds. Similarly, as for testosterone sensitive chemical bursectomy, avian embryos were exposed to high levels of estrogens via *in ovo* injection. Estradiol administration caused demasculinization in male Japanese quails leading to the loss of sexual behaviour such as head mounting and grabbing, typical male response to females [159]. Further, estradiol implants were applied to gonadsectomized males and females in Japanese quails shortly after hatch. In such females, sexual behaviour was suppressed in the estradiol treated group compared to controls. Surprisingly, estradiol treated males did not lose the copulatory behaviour. In addition, no effect was observed, when the estradiol implants were applied in adult birds. These results indicate that estrogen exposure cause demasculinization only if the treatment is applied before hatching [160].

Of course, one has to keep in mind that these effects are, most likely, pharmacological effects caused by estradiol overdose.

ARKO$^{-/-}$ hens and roosters exhibited very similar phenotypes, and it was not easily possible to distinguish between the sexes by eye. Nevertheless, it was noted that ARKO$^{-/-}$ roosters grew longer tail feathers than ARKO$^{-/-}$ hens indicating that this pattern has to be sex-specific but not androgen-dependent. Interestingly, one publication examined the lack of tail feathers occasionally observed in Honghan chicken. This study revealed that the lack is linked to mutation of the Z chromosome [141]. These findings are in line with our suggestion that the growing of tail feathers is driven by the sex chromosome and not androgen-dependent.

6.4. Testosterone treatment did not cause regression of the bursa of Fabricius in ARKO$^{-/-}$ embryos

The Bursa of Fabricius is a lymphoid organ vital for B-cell development. During sexual maturation, the bursa involutes and regresses entirely with the onset of sexual maturity [161]. The role of sex hormones in bursa involution is still not very well understood, and it is believed that their function might be essential [88]. It was reported that dipping eggs into a high dosage of testosterone results in chemical bursectomy and subsequent B-cell depletion [162]. The bursa involution caused by testosterone represents a great possibility to prove the successful knock-out of the AR on functional level *in vivo* in males and females.

Our results showed that after testosterone dipping, bursa of WT embryos regressed, while bursa of ARKO$^{-/-}$ embryos became unaffected. It was not surprising after all that B-cell also exhibited poor colonization in WT treated bursae since the stroma and B-cell follicles did not develop.

Data from our experiment and published literature indicate [89, 90, 91] that the bursa is sensitive for androgens and brings up questions, whether the bursa of ARKO$^{-/-}$ involutes during sexual maturation. Of course, one has to keep in mind that concentrations affecting the eggs during our experiment were most likely much higher than the natural levels of testosterone. Nevertheless, the lack of androgens does affect bursa development in adult birds. For instance, a castration in Japanese quails arrested the bursa involution [163]. Similar effect Glick *et al.* observed in chickens that caponization caused an increase of the bursa size in comparison to controls [162].

Further questions, however, arise: Is the bursa of Fabricius sensitive for androgens? Can other steroid hormones influence the bursa as strongly as androgens? These questions are particularly interesting in light of the experiments of Ylikomi *et al.* where the bursa involution in chicken embryos was induced by estradiol and progesterone and will be answered in future experiments using AR knockout chickens [164].

6.5. Knock-out of the androgen receptor did not influence the PBMCs composition in ARKO male chickens

It is well known that steroid hormones possess immunomodulatory abilities [165]. When the ARKO mice models were established, the impact on the immune system was as well examined [13]. Therefore, various PMBCs (Peripheral blood mononuclear cells) populations at different time points were examined to investigate the influence of AR/androgen system in ARKO$^{-/-}$ chickens.No significant dif- ference of the analyzed PBMCs populations was found between ARKO$^{-/-}$ and WT roosters. This result is surprising because the effects of androgens, respectively testosterone, on the immune system of males were already reported by several studies in chickens. Arstila *et al.* reported effect of androgen administration on $\gamma\delta$ T-cells. The increased absolute number of blood $\gamma\delta$ T-cell subset correlated with higher levels of testosterone present during sexual maturation of roosters. The same expansion effect of $\gamma\delta$ T-cells was observed after testosterone administration to hens [166]. Unfortunately, no further studies were later reported confirming these observations. On the opposite side, reduced levels of testosterone, for instance after caponization, seem to affect the thymus development. Mashaly *et al.* reported a decreased cell-mediated immune response and reduced size of the thymus in immature caponized roosters [167].

In our experiments, ARKO $^{-/-}$ females exhibited significant differences in all measured PBMCs populations. B-cells, monocytes, $\alpha\beta$ T-cells and $\gamma\delta$ T-cells were significantly decreased in comparison to WT females. Previous studies did not analyze the role of a dysfunctional androgen environment in females. Nevertheless, sex differences in the immune response to infection or in antibody levels were described in chickens as well.

Leitner *et al.* reported much higher mortality rates in males after infection with various agents, which was explained by an earlier peak of antibody response in females than in males [168].

Furthermore, it is well known in humans and mice that the functionality of the immune system differs between the sexes [169]. Women are more susceptible to autoimmune diseases and show a difference in innate and adaptive immune responses compared to men [170]. Therefore, it can be assumed that sex steroid hormones have to play a role in females, which can administrate in chickens as well.

Our results indicate that there might be an effect of impaired AR/androgen signaling on absolute counts of PBMCs populations but a deeper investigation is be necessary to confirm it.

6.6. Outlook

Generation of ARKO$^{-/-}$ chicken allows new insight into chicken sexual development. Further experiments aim to measure hormone blood levels, histology of reproductive organs, and deeper investigation of the behavior of ARKO$^{-/-}$ chicken. However, a complete lack of AR activity also allows studying other body systems besides the reproductive system. In humans or mice, AR/androgen system was also involved in various processes starting from fat and glucose metabolism [11, 12] ending with cardiovascular [25, 26], or immune system [13]. For instance, in chicken, the faith of the bursa of Fabricius and its involution would be interesting to investigate. My preliminary data indicate that the size of the bursa is bigger in ARKO$^{-/-}$ than in WT chicken at the age of 19th weeks. However, these results have to be statistically analyzed and need further investigation. This investigation would deepen our understanding of the mechanisms of bursa involution. Although, one has to keep in mind that other sex hormones next to androgens, such as estrogens, might be involved in the process. Obviously, it would be great to observe the bursa development in estrogen receptor knock-out chicken.

In the future, an attractive approach would be to generate conditional knock-out enabling to target cells of different organs as testes, ovaries, or brain. In mice, for instance, these studies underlined the importance of AR in Sertoli cells and revealed that AR is not essential in germ cell development [4].

Furthermore, our AR reporter chicken line can help with studying AR expression in different tissues during embryonic development and sexual maturity without the need to use any antibodies. Combined knowledge from both chicken lines can help to understand the role of androgen in chickens.

7. Conclusion

It is well accepted that androgens are essential in sex development in most species. In mice and humans, it has been proposed that dysfunctional androgen signaling is caused by mutations of the androgen receptor. With the progress of gene-editing technology, these observations have been confirmed not only by sequencing but as well by the generation of androgen receptor knock-out mice. In birds, for a long time, the generation of genetically modified organisms was not possible. Besides the unique reproductive system, the chicken embryo consists already of about 50 000 cells at the time the egg is layed, which does not allow microinjection as for example in mice. Later on, the development of a cell culture system for chicken primordial germ cells (precursors for sperm and eggs) created a powerful tool for chicken transgenesis. Furthermore, the establishment of a conditioned medium provided a simplified method of primordial germ cell (PGCs) culturing and allows more easy gene editing in chickens.

In this study, I have generated an androgen receptor knockout chicken (ARKO) chicken by editing chicken primordial germs cells using the CRISPR/Cas9 system. Initially, ARKO was introduced in PGCs, which, after transfer into recipient embryos differentiate in sperms carrying the desired androgen receptor knock-out. By breeding germline chimeras, ARKO heterozygous chickens were generated. ARKO$^{+/-}$ did not exhibit any specific phenotype different from WT chickens, indicating that only one copy of AR is sufficient to maintain the sexual development as well as normal fertility. Further breeding gave rise to ARKO homozygous chickens. ARKO$^{-/-}$ roosters did not express any sexual secondary sex characteristic or behavior. Typical characteristics such as a large comb, wattles, or spur were not present, indicating that these organs are androgen-regulated. ARKO$^{-/-}$ roosters suffered from infertility and did not produce any sperms. In hens, a similar phenotype was observed, and they were almost sexually indifferent to heterozygous ARKO males. Hens also did not exhibit any female typical sex characteristics such as a comb, and no egg-laying was observed. Interestingly, ARKO$^{-/-}$ roosters grew longer tail feathers than hens indicating that this has to be sex-specific but not androgen-dependent.

As proof of the successful knock-out, we have performed a simple experiment by dipping ARKO and WT eggs into testosterone, which induces chemical bursectomy. Histological staining showed that inner stroma tissue with B-cell follicles did not develop. In contrast, bursae of ARKO$^{-/-}$ embryos appeared as WT bursae with

developed B-cell follicles. Several publications, as well as our research, indicate the sensitivity of the bursa of Fabritius for steroid hormones. Therefore, in the future, the development of the bursa should be closely studied after hatching and during sexual maturation.

Based on findings in mice and humans, it was also examined whether the impaired AR/androgen signaling will affect the immune system in chicken. By flow cytometry, the peripheral blood mononuclear cells (PBMC) composition was measured. ARKO$^{-/-}$ males did not exhibit any difference compared to WT chicken, while in ARKO$^{-/-}$ females, absolute counts of the stained cell populations were significantly decreased.

Furthermore, different strategies for generation of androgen reporter chicken were examined. This approach aims for tagging of the androgen receptor *in vivo* allowing visualization without the need of using antibodies. Initially, a reporter assay was designed to test whether the proposed modification of AR won´t interfere with AR signaling. Our results showed that AR-flag tag and AR-mCherry fusion proteins obtain the ability of signal transduction *in vitro*. Then, we have performed targeting via homologous directed repair and CRISPR/Cas9 system. This strategy was not successful, most likely because of the weak expression of AR in PGCs. Nevertheless, successful editing was detected after transfection with ssODN and CRISPR/Cas9. In the future, PGCs will be transfected with ssDNA constating mCherry for AR tagging. Due to time limitation, I could not establish the AR reporter line itself.

In this study, I have generated an ARKO chicken model confirming that androgens have an enormous impact on sexual development of male and female chicken, despite the fact that sexual differentiation might be cell-autonomous in birds. Furthermore, we have examined different strategies for generation AR reporter chicken line. My research aims for better understanding of roles of androgen in chicken sexual development.

A. Appendix

A.1. List of chemicals and Reagents

1. ThermoFischer Scientific, Waltham, USA
2. CLN GmbH, Freising, Germany
3. Carl Roth GmbH & Co. KG, Karlsruhe, Germany
4. Merck KGaA, Darmstadt, Germany
5. Preprotech, Hamburg, Germany
6. New England Biolabs, Ipswich, USA
7. R&D Systems, Minneapolis, USA
8. Applichem, Darmstadt, Germany
9. Fisher Scientific GmbH, Schwerte, Germany
10. Hilgenberg, Malsfeld, Germany
11. Conrad Electronic, Hirschau, Germany
12. Sarstedt, Nümbrecht, Germany
13. Siepmann GmbH, Herdecke, Germany
14. neoLab Migge GmbH, Heidelberg, Germany
15. VWR International GmbH, Darmstadt Germany
16. Lonza, Basel, Schwitzerland
17. Promega GmbH, Mannheim, Germany
18. Analytik Jena GmbH, Jena, Germany
19. Solis Biodyne, Tartu, Estonia
20. Eurofins Genomics, Ebersberg, Germany
21. Integrated DNA Technologies, Inc. , Iowa, USA

22. Brüterei Thole, Bösel, Germany
23. S.C. Johnson & Son, Wisconsin, USA
24. Biozym Scientific GmbH, Wien, Austria
25. Biozol Diagnostica Vetrieb GmbH, Eching, Germany
26. Medite GmbH, Burgdorf, Germany

A.2. Laboratory instruments in alphabetical order

Equipment	Producer
Centrifuge 5810	Eppendorf AG
Dremmel Tool ® 3000-15	Conrad Electronic GmbH, Hirschau
Egg candeling light	Siepmann GmbH, Herdecke
Electrophoresis system (source and champer)	Bio-Rad, Hercules, USA
Flow cytometer Attune NxT	ThermoFischer Scientific, Waltham, USA
Gel imaging system Quantum	Vilber GmbH, Eberhardzell
Homogenizer SpeedMill Plus	Analytikjena GmbH, Jena
Incubator HEKA-Olymp	HEKA Brutgeräte GmbH, Rietberg
Incubator	ThermoFischer Scientific, Waltham, USA
Incubator Procon	Grumbach Brutgeräte GmbH, Asslar
Laminar flow hood	Heraeus Holding GmbH, Hanau
Microcentrifuge 5415R	Eppendorf AG, Cologne
Microscope Leica DMIL Camera DF340XF	Leica GmbH
Microscope	Zeiss AG, Oberkochen
NanoDrop ND-100	Peqlab GmbH, Erlangen
Neubauer improved chamber	Brand, Wertheim
PCR Workstation Pro	Peqlab GmbH, Erlangen
Plate reader FLUOstar Omegra	BMG LABTECH
Thermal Cycler	Bio-Rad, Hercules, USA
Thermal Cycler	Peqlab GmbH, Erlangen
Vortex Mixer	VELP Scientifica Srl, Usmate,Italy

A.3. Statistics for testosterone induced bursectomy in R studio

Here we have an example data, how the statistic was performed in the R studio. In this case WT and homozygous fro the treated group was compared.

1. **Normality test**

```
> shapiro.test(WT$WT)

Shapiro-Wilk normality test

data:  WT$WT W = 0.87492, p-value = 0.114

> shapiro.test(Homo$Homo)

Shapiro-Wilk normality test

data:  Homo$Homo W = 0.95146, p-value = 0.7259
```

2. **F-test to compare two variances**

```
> var.test(WT$WT, Homo$Homo)

data:  WT$WT and Homo$Homo

F = 2.3017, num df = 8, denom df = 7, p-value = 0.2887

alternative hypothesis:  true ratio of variances is not equal
to 1, 95 percent confidence interval:

0.4437334 8.9826137

sample estimates:

ratio of variances 2.140223
```

3. **Two sample t-test**

```
> t.test(WT$WT, Homo$Homo, alternative = c("two.sided"), mu =
0, paired = FALSE, var.equal = TRUE, conf.level = 0.95)

Two Sample t-test

data:  WT$WT and Homo$Homo

t = -2.6316, df = 15, p-value = 0.01887

alternative hypothesis:  true difference in means is not equal
to 0,95 percent confidence interval:

-0.56315386 -0.05913421
```

```
sample estimates:
mean of x mean of y
0.7567564 1.0860541
```

List of Figures

List of Tables

Glossary

tfm Testicular feminization.

17βHSD 17 β-hydroxysteroid dehydrogenase.

AIS Androgen insensitivity syndrome.

AMH Anti-Müllerian hormone.

Amhr2 Anti-Müllerian hormone receptor-2.

AR Androgen receptor.

ARE Androgen response element.

ARKO Androgen receptor knock-out.

ARKO $^{+/-}$ Androgen receptor knock-out heterozygous.

ARKO $^{-/-}$ Androgen receptor knock-out homozygous.

ARKO LO Androgen receptor knock-out loop out.

ATP Adenosine triphosphate.

BSA Bovine serum albumine.

$CaCl_2$ Calcium Chloride.

CAIS Complete androgen insensitivity syndrome.

Cas CRISPR associated.

CEF Chicken embryonic fibroblasts.

CH_3COONa Sodium acetate.

CO_2 Carbon dioxide.

CRISPaint CRISPR-assisted insertions tagging.

CRISPR/Cas9 Clustered regulatory interspaced short palindromic repeats.

crRNA CRISPR-derived RNA.

CYP19A1 Aromatase.

DAB 3,3-Diaminobenzidine.

DAPI 4,6-diamidino-2-phenylindole.

DBD DNA-binding domain.

DBS Double strand break.

DHT Dihydrotestosterone.

DMEM Dulbecco's Modified Eagle's Medium.

DMRT1 Doublesex and mab-3-related transcription factor 1.

DMSO Dimethylsulfoxid.

DTT Dithiothreitol.

E Estrogen.

ED Embryonic day.

EDTA Ethylenediaminetetraacetic acid.

eGFP Enhanced GFP.

EtOH Ethanol.

FACS Fluorescence-activated cell sorting.

FBS Fetal bovine serum.

FET1 Female-expressed transcript.

FGF Fibroblast growth factor.

FOXL2 Forkhead Box L2.

FSH Follicle-stimulating hormone.

GA Gibson Assembly.

GC Glycine-Serine.

GFP Green fluorescent protein.

GnRH Gonadotropin-releasing hormone.

H_2O Water.

H_2O_2 Hydrogen peroxide.

H&H Hamburger and Hamilton.

HCl Hydrochloric acid.

HDR Homologous directed repair.

HR Homologous region.

HSP Heat shock protein.

Ig Immunoglobulin.

IgG Immunoglobulin G.

KCl Potassium chloride.

KH_2PO_4 Dipotassium hydrogenphosphate.

KO Knock-out.

LARKO Leydig cells knock-out.

LB Lysogeny Broth.

LBD Ligand-binding domain.

LH Luteinizing hormone.

LSL Lohmann´s Selected Leghorn Classic.

MgCl Magnesium chloride.

MMTV LTR Murine mammary tumor virus long terminal repeat.

$Na_2HPO_4x2H_2O$ Disodium hydrogen phosphate dihydrate.

NaCl Sodium chloride.

NaOH Sodium hydroxide.

NTD N-terminal domain.

P Progesterone.

PAIS Partial androgen insensitivity syndrome.

PAM Protospacer adjacent motif.

PBS Phosphate-buffered saline.

PCR Polymerase chain reaction.

PFA Paraformaldehyde.

PGCs Primordial germ cells.

PMBC Peripheral blood mononuclear cells.

PNK Polynucleotide kinase.

RPMI Roswell Park Memorial Institute Medium.

SCARKO Knock-out of AR in Sertoli cells.

SDS Sodium dodecyl sulfate.

sgRNA Single-chain guide RNA.

SNPs Single-nucleotide polymorphism.

SOC Super Optimal broth with Catabolites repression.

SOX9 Sex determining region Y-box 9.

ssDNA Single stranded DNA.

ssODN Single stranded oligonucleotide.

STM Sucrose-Tris-Magnesium chloride.

T Testosterone.

TAE Tris-Acetate-EDTA.

TALENs Transcription Activator-Like Effector Nucleases.

TBE Tris-Borate-EDTA.

TEN Tris-EDTA-NaCl.

tracrRNA transactivating RNA.

Tris-HCl Tris(hydroxymethyl)aminomethane hydrochloride.

WT Wildtype.

µ Micro.

µg Microgramm.

µl Microliter.

α Alpha.

β Beta.

γ Gamma.

δ Delta.

Bibliography

[1] A. Cutting, J. Chue, and C. Smith. "Just how conserved is vertebrate sex determination?" In: *Dev Dyn* 4 (2013), pp. 380–7.

[2] G. F. Weinbauer and E. Nieschlag. *Testosterone*. Methods in Molecular Biology. Springer, Berlin, Heidelberg, 1990. ISBN: 9783662008140.

[3] C. A. Quigley, A. D. Bellis, K. B. Marschke, M. K. el-Awady, E. M. Wilson, and F. S. French. "Androgen receptor defects: historical, clinical, and molecular perspectives." In: *Endocr Rev* 16 (1995), pp. 271–321.

[4] K. A. Walters, U. Simanainen, and D. J. Handelsman. "Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models." In: *Hum Reprod Update* 16 (2010), pp. 543–58.

[5] D. Zhao, D. McBride, S. Nandi, H. A. McQueen, M. J. McGrew, P. M. Hocking, P. D. Lewisa, H. M. Sang, and M. Clinton. "Somatic sex identity is cell autonomous in the chicken." In: *Nature* 464 (2010), pp. 237–242.

[6] W. W. He, M. V. Kumar, and D. J. Tindall. "A frame-shift mutation in the androgen receptor gene causes complete androgen insensitivity in the testicular-feminized mouse." In: *Nucleic Acids Res* 19 (1991), pp. 2373–2378.

[7] S. Kato. "Androgen receptor structure and function from knock-out mouse." In: *Clin Pediatr Endocrinol* 11 (2002), pp. 1–7.

[8] R. W. Holdcraft and R. E. Braun. "Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids." In: *Development* 131 (2004), pp. 459–67.

[9] S. Yeh, M. Tsai, Q. Xu, X. Mu, H. Lardy, K. Huang, H. Lin, S. Yeh, S. Altuwaijri, X. Zhou, L. Xing, B. F. Boyce, M. Hung, S. Zhang, L. Gan, and C. Chang. "Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues." In: *Proc Natl Acad Sci USA* 99 (2002), pp. 13498–503.

[10] A. J. Notini, R. A. Davey, J. F. McManus, K. L. Bate, and J. D. Zajac. "Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model." In: *J Mol Endocrinol.* 35 (2005), pp. 547–55.

[11] H. Lin, Q. Xu, S. Yeh, R. Wang, J. D. Sparks, and C. Chang. "Insulin and Leptin Resistance With Hyperleptinemia in Mice Lacking Androgen Receptor." In: *Diabetes* 6 (2005), pp. 1717–1725.

[12] T. Yanase, W. Fan, K. Kyoya, L. Min, R. Takayanagi, S. Kato, and H. Nawata. "Androgens and metabolic syndrome: lessons from androgen receptor knock out (ARKO) mice." In: *J Steroid Biochem Mol Biol* 109 (2008), pp. 254–7.

[13] J. Lai, K. Lai, W. Zeng, K. Chuang, S. Altuwaijri, and C. Chang. "Androgen Receptor Influences on Body Defense System via Modulation of Innate and Adaptive Immune Systems." In: *Am J Pathol* 181 (2012), pp. 1504–1512.

[14] T. Sato, T. Matsumoto, H. Kawano, T. Watanabe, Y. Uematsu, K. Sekine, T. Fukuda, K. Aihara, A. Krust, T. Yamada, Y. Nakamichi, Y. Yamamoto, T. Nakamura, K. Yoshimura, T. Yoshizawa, D. Metzger, P. Chambon, and S. Kato. "Brain masculinization requires androgen receptor function." In: *Biochim Biophys Acta* 101 (2004), pp. 1673–8.

[15] Q. Wang, J. Pi, A. Pan, J. Shen, and L. Qu. "A novel sex-linked mutant affecting tail formation in Hongshan chicken." In: *Sci Rep* 7 (2017).

[16] J. Ioannidis, G. Taylor, D. Zhao, L. Liu, A. Idoko-Akoh, D. Gong, R. Lovell-Badge, S. Guioli, M. McGrew, and M. Clinton. "Primary sex determination in birds depends on DMRT1 dosage, but gonadal sex does not determine adult secondary sex characteristics." In: *PNAS* 10 (2021), p. 118.

[17] R. Greger and U. W. (Eds.) *Comprehensive Human Physiology*. From Cellular Mechanisms to Integration. Springer, 1996. ISBN: 9783642609466.

[18] E. S. E. Hafez and B. H. (Eds). *Reproduction in Farm Animals*. Wiley-Blackwell, 2013. ISBN: 9781118710289.

[19] C. Biol. "Spermatogenesis". In: *J Steroid Biochem Mol Biol* 27 (2017), pp. 988–994.

[20] J. Lim, G. J. Maher, G. D. H. Turner, S. T. W. Dudka-Ruszkowska, E. R. Meyts, A. Goriely, and A. O. M. Wilkie. "Selfish spermatogonial selection: evidence from an immunohistochemical screen in testes of elderly men." In: *PLoS One* 7 (2012), pp. 385–422.

[21] F. Sánchez and J. Smitz. "Molecular control of oogenesis." In: *Biochim Biophys Acta* 12 (2012), pp. 1896–912.

[22] K. Chang, S. Altuwaijri, G. Li, J. Lai, C. Chu, K. Lai, H.Lin, J. Hsu, P. Keng, M. Wu, and C. Chang. "Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor." In: *J Exp Med* 206 (2009), pp. 1181–99.

[23] G. S. Ashcroft and S. J. Mills. "Androgen receptor-mediated inhibition of cutaneous wound healing." In: *J Exp Med* 110 (2002), pp. 615–24.

[24] S. Altuwaijri, K. Chuang, K. Lai, J. Lai, H. Lin, F. M. Young, A. Bottaro, M. Tsai, W. Zeng, H. Chang, S. Yeh, and C. Chang. "Susceptibility to autoimmunity and B cell resistance to apoptosis in mice lacking androgen receptor in B cells." In: *Mol Endocrinol.* 23 (2009), pp. 444–53.

[25] Y. Li, I. Kishimoto, Y. Saito, M. Harada, K.Kuwahara, T. Izumi, I. Hamanaka, N. Takahashi, R. Kawakami, and K. Tanimoto. "Androgen Contributes to Gender-Related Cardiac Hypertrophy and Fibrosis in Mice Lacking the Gene Encoding Guanylyl Cyclase-A." In: *Endocrinology* 145 (2004), pp. 951–958.

[26] Y. Ikeda, K. Aihara, T. Sato, M. Akaike, M. Yoshizumi, Y. Suzaki, Y. Izawa, M. Fujimura, S. Hashizume, M. Kato, S. Yagi, T. Tamaki, H. Kawano, T. Matsumoto, H. Azuma, S. Kato, and T. Matsumoto. "Androgen Receptor Gene Knockout Male Mice Exhibit Impaired Cardiac Growth and Exacerbation of Angiotensin II-induced Cardiac Fibrosis." In: *J Biol Chem* 208 (2005), pp. 29661–29666.

[27] C. Heinlein and C. Chang. "Androgen receptor (AR) coregulators: an overview." In: *Endocr Rev* 23 (2002), pp. 175–200.

[28] Y. O. H. Katoh and G. Yamada. "Cloning and expression analysis of androgen receptor gene in chicken embryogenesis." In: *FEBS Lett* 580 (2006), pp. 1607–15.

[29] A. Roy, Y. Lavrovsky, C. Song, S. Chen, M. Jung, N. Velu, B. Bi, and B. Chatterjee. "Regulation of androgen action." In: *Vitam Horm* 55 (1999), pp. 309–52.

[30] E. P. Gelmann. "Molecular biology of the androgen receptor." In: *J Clin Oncol* 20 (2002), pp. 3001–15.

[31] T. Matsumoto, M. Sakari, M. Okada, A. Yokoyama, S. Takahashi, A. Kouzmenko, and S. Kato. "The Androgen Receptor in Health and Disease." In: *Annu. Rev. Physiol* 75 (2013), pp. 201–224.

[32] P. Lonergan and D. Tindall. "Androgen receptor signaling in prostate cancer development and progression." In: *J. Carcinog.* 10 (2011), p. 20.

[33] M. G. R. Davey. "Androgen Receptor Structure, Function and Biology: From Bench to Bedside." In: *Clin Biochem Rev* 37 (2016), pp. 3–15.

[34] D. T. M. Grossmann H. Huang. "Androgen receptor signaling in androgen-refractory prostate cancer." In: *J Natl Cancer Inst* 37 (2011), pp. 1687–97.

[35] M. F. Lyon and S. G. Hawkes. "X-linked gene for testicular feminization in the mouse." In: *Nature* 227 (1970), pp. 1217–9.

[36] R. D. Jones, P. J. Pugh, J. Hall, K. S. Channer, and T. H. Jones. "Altered circulating hormone levels, endothelial function and vascular reactivity in the testicular feminised mouse." In: *Eur J Endocrinol* 148 (2003), pp. 111–20.

[37] C. W. Bardin and L. P. Bullock. "Proceedings: Testicular feminization: studies of the molecular basis of a genetic defect." In: *J Invest Dermatol* 63 (1974), pp. 75–84.

[38] X. Zhou. "Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice." In: *J Androl* 31 (2010), pp. 235–43.

[39] K. D. Gendt, J. V. Swinnen, P. T. K. Saunders, L. Schoonjans, M. Dewerchin, A. Devos, K. Tan, N. Atanassova, F. Claessens, C. Lécureuil, W. Heyns, P. Carmeliet, F. Guillou, R. M. Sharpe, and G. Verhoeven. "A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis." In: *Proc Natl Acad Sci USA* 101 (2004), pp. 1327–32.

[40] K. A. Walters, C. M. Allan, M. Jimenez, P. R. Lim, R. A. Davey, J. D. Zajac, P. Illingworth, and D. J. Handelsman. "Female mice haploinsufficient for an inactivated androgen receptor (AR) exhibit age-dependent defects that resemble the AR null phenotype of dysfunctional late follicle development, ovulation, and fertility." In: *Endocrinology* 148 (2007), pp. 3674–3684.

[41] Y. Hu, P. Wang, S. Yeh, R. Wang, C. Xie, Q. Xu, X. Zhou, H. Chao, M. Tsai, and C. Chang. "Subfertility and defective folliculogenesis in female mice lacking androgen receptor." In: *Proc Natl Acad Sci USA* 101 (2004), pp. 11209–11201.

[42] Q. Xu, H. Lin, S. Yeh, I. Yu, R. Wang, Y. Chen, C. Zhang, S. Altuwaijri, L. Chen, K. Chuang, H. Chiang, S. Yeh, and C. Chang. "Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells." In: *Endocrine* 32 (2007), pp. 96–106.

[43] P. Jeyasuria, Y. Ikeda, S. P. Jamin, L. Zhao, D. G. D. Rooij, A. P. N. Themmen, R. R. Behringer, and K. L. Parker. "Cell-specific knockout of steroidogenic factor 1 reveals its essential roles in gonadal function." In: *Mol Endocrinol* 18 (2004), pp. 1610–1619.

[44] P. Lim, M. Robson, J. Spaliviero, K. J. McTavish, M. Jimenez, J. D. Zajac, D. J. Handelsman, and C. M. Allan. "Sertoli cell androgen receptor DNA binding domain is essential for the completion of spermatogenesis." In: *Endocrinology* 150 (2009), pp. 4755–4765.

[45] C. Chang, Y. Chen, S. Yeh, Q. Xu, R. Wang, F. Guillou, H. Lardy, and S. Yeh. "Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells." In: *Proc Natl Acad Sci USA* 101 (2004), pp. 6876–6881.

[46] S. Kerkhofs, S. Denayer, A. Haelens, and F. Claessens. "Androgen receptor knockout and knock-in mouse models." In: *J Mol Endocrinol* 32 (2009), pp. 11–7.

[47] M. Tsai, S. Yeh, R. Wang, S. Yeh, C. Zhang, H. Lin, C. Tzeng, and C. Chang. "Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells." In: *Proc Natl Acad Sci USA* 103 (2006), pp. 18975–18980.

[48] C. A. Smith. "Sex Determination in Birds: HINT s from the W Sex Chromosome?." In: *Sexual Development* 1 (2007), pp. 279–285.

[49] P. Koopman, J. Gubbay, N. Vivian, P. Goodfellow, and R. Lovell-Badge. "Male development of chromosomally female mice transgenic for Sry." In: *Curr Protoc Microbiol* 351 (1991), pp. 117–21.

[50] P. Parma and O. Radi. "Molecular mechanisms of sexual development." In: *Sex Dev* 6 (2012), pp. 7–17.

[51] H. Ellegren, L. Hultin-Rosenberg, B. Brunström, L. Dencker, K. Kultima, and B. Scholz. "Faced with inequality: chicken do not have a general dosage compensation of sex-linked genes." In: *BMC Biology* 5 (2007), p. 40.

[52] C. A. Smith. "Sex determination in birds: a review." In: *Emu* 110 (2010), pp. 364–377.

[53] T. Sasanami(Ed.) *Avian Reproduction*. Advances in Experimental Medicine and Biology. Springer Nature Singapore, 2017. ISBN: 9789811039751.

[54] C. A. Smith and A. H. Sinclair. "Sex determination:insights from the chicken." In: *BioEssays* 2 (2004), pp. 120–132.

[55] C. A. Smith, K. N. Roeszler, T. Ohnesorg, D. M. Cummins, P. G. Farlie, T. J. Doran, and A. H. Sinclair. "The avian Z-linked gene DMRT1 is required for male sex determination in the chicken." In: *Nature* 461 (2009), pp. 267–271.

[56] C. G. Scanes(Ed.) *Sturkie's Avian Physiology (Sixth Edition)*. Academic Press, 2015. ISBN: 9780124071605.

[57] M. J. Fennell and C. G. Scanes. "Inhibition of growth in chickens by testosterone, 5α-dihydrotestosterone and 19- nortestosterone." In: *Poult Sci* 71 (1992), pp. 357–366.

[58] M. H. Henry and W. H. Burke. "The effects of in ovo administration of testosterone or an antiandrogen on growth of chick embryos and embryonic muscle characteristics." In: *Poult Sci* 78 (1999), pp. 1006–13.

[59] G. K. Symeon, M. Charismiadou, F. Mantis, I. Bizelis, A. Kominakis, and E. Rogdakis. "Effects of caponization on fat metabolism-related biochemical characteristics of broilers." In: *J Anim Physiol Anim Nutr (Berl)* 97 (2013), pp. 162–9.

[60] J. Calik, S. Świątkiewicz, J. Obrzut, K. Połtowicz, and J. Krawczyk. "Effects of Caponization on Growth Performance and Meat Physicochemical Properties of Crossbred Chickens." In: *Ann Anim Sci* 20 (2020), pp. 1509–1525.

[61] K. Severin, T. Mašek, D. Horvatek, D. Konjević, Z. Janicki, Ž. Cvrtila, L. Kozačinski, L. Hadžiosmanovič, and R. Barić-Rafaj. "The effects of castration on the growth parameters, carcass yield and meat chemical composition of intensively reared Common Pheasant (Phasianus colchicus colchicus L.)." In: *Ital J Anim Sci* 6 (2006), pp. 213–219.

[62] M. R. Burke, E. Adkins-Regan, and J. Wade. "Laterality in syrinx muscle morphology of the Japanese quail (Coturnix japonica)." In: *Physiol Behav* 90 (2007), pp. 682–6.

[63] R. S. Hartley, M. S. Chinn, and N. F. Ullrich. "Left syringeal dominance in testosterone-treated female canaries." In: *Neurobiol Learn Mem* 67 (1997), pp. 248–53.

[64] F. Nottebohm, T. M. Stokes, and C. M. Leonard. "Central control of song in the canary, Serinus canarius." In: *J Comp Neurol* 165 (1976), pp. 457–86.

[65] J. Wade and L. Buhlman. "Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in zebra finches." In: *J Comp Neurol* 426 (2000), pp. 3154–164.

[66] S. E. Allan and R. A. Suthers. "Lateralization and motor stereotypy of song production in the brown-headed cowbird." In: *J Neurobiol* 25 (1994), pp. 1154–66.

[67] S. F. Godsave, R. Lohmanna, R. P. M. Vloeta, and M. Gahr. "Androgen receptors in the embryonic zebra finch hindbrain suggest a function for maternal androgens in perihatching survival." In: *J Comp Neurol* 453 (2002), pp. 57–70.

[68] S. L. Veney and J. Wade. "Steroid receptors in the adult zebra finch syrinx: a sex difference in androgen receptor mRNA, minimal expression of estrogen receptor alpha and aromatase." In: *Gen Comp Endocrinol* 136 (2004), pp. 192–9.

[69] T. Shimmura, M. Tamura, S. Ohashi, A. Sasaki, T. Yamanaka, N. Nakao, K. Ihara, S. Okamura, and T. Yoshimura. "Cholecystokinin induces crowing in chickens." In: *Sci Rep* 8 (2019), p. 9.

[70] M. Konishi. "The Role of Auditory Feedback in the Vocal Behavior of the Domestic Fowl." In: *J Ethol* 20 (1963), pp. 349–367.

[71] D. P. Quiring. "Transplantation of testes (by A. A. Bethold)." In: *Bull Hist Med* 16 (1944), pp. 399–401.

[72] K. K. Soma. "Testosterone and Aggression: Berthold, Birds and Beyond." In: *J Neuroendocrinol* 18 (2006), pp. 543–551.

[73] P.Makler, M.Kreith, and E.Willis. "An analysis of testosterone-induced crowing in young domestic cockerels." In: *Anim Behav* 10 (1962), pp. 48–54.

[74] J. Hamilton. "Precocious masculine behavior following administration of synthetic male hormone substances." In: *Endocrinology* 23 (1938), pp. 53–57.

[75] G. Noble and A. Zitrin. "Induction of mating behaviour in male and female chicks following injection of sex hormones." In: *Endocrinology* 30 (1942), pp. 327–334.

[76] Y. Yazaki, T. Matsushima, and K. Aoki. "Testosterone Modulates Calling Behavior in Japanese Quail Chicks." In: *Zool Sci* 14 (1997), pp. 219–225.

[77] M. Gahr. "Sexual differentiation of the vocal control system of birds." In: *Adv Genet* 59 (2007), pp. 67–105.

[78] E. Vallet, M. Kreutzer, and M. Gahr. "Sexual differentiation of the vocal control system of birds." In: *J Ethol* 102 (1996), pp. 617–628.

[79] S. D. Brown and S. W. Bottjer. "Testosterone-induced changes in adult canary brain are reversible." In: *J Neurobiol* 24 (2007), pp. 627–40.

[80] M. Gahr. "How Hormone-Sensitive Are Bird Songs And What Are The Underlying Mechanisms?" In: *Acta Acust united Ac* 14 (2014), pp. 705–718.

[81] M. C. Ko, C. Frankl-Vilches, A. Bakker, and M. Gahr. "The Gene Expression Profile of the Song Control Nucleus HVC Shows Sex Specificity, Hormone Responsiveness, and Species Specificity Among Songbirds." In: *Front Neurosci* 15 (2021).

[82] F. A. Beach and N. G. Inman. "Effects of castration and androgen replacement on mating in male quail." In: *Proc Natl Acad Sci USA* 54 (1965), pp. 1426–31.

[83] J. A. Wilson and B. Glick. "Ontogeny of mating behavior in the chicken." In: *Am J Physiol* 218 (1970), pp. 951–5.

[84] J. C. Wingfield. "Androgens and Mating Systems: Testosterone-induced Polygyny in Normally Monogamous Birds." In: *Auk* 101 (1984), pp. 665–671.

[85] E. K. Adkins, J. J. Boop, D. L. Koutnik, J. B. Morris, and E. E. Pniewski. "Further evidence that androgen aromatization is essential for the activation of copulation in male quail." In: *Physiol Behav* 24 (1980), pp. 441–6.

[86] M. J. Walters and C. F. Harding. "The effects of an aromatization inhibitor on the reproductive behavior of male zebra finches." In: *Horm Behav* 22 (1988), pp. 207–18.

[87] B. A. Schlinger and G. V. Callard. "Aromatization mediates aggressive behavior in quail." In: *Gen Comp Endocrinol* 79 (1990), pp. 39–53.

[88] K. A. Schat, B. Kaspers, and (. P. Kaiser. *Avian Immunology*. Structure of the Avian Lymphoid System. Academic Press, 2014. ISBN: 9780123969651.

[89] J. Wilson, N. Mitlin, and B. Glick. "Passage of Testosterone-4-$C^{1}4$ from the Egg Shell to the Albumin." In: *Poult Sci J* 50 (1977), pp. 655–656.

[90] B. Glick. "The influence of dipping eggs in male hormone solutions on lymphatic tissue and anti- body response of chickens." In: *Poult Sci J* 69 (1961), pp. 984–985.

[91] I. Olah, B. Glick, and I. Törö. "Bursal development in normal and testosterone-treated chick embryos." In: *Poult Sci* 65 (1986), pp. 574–88.

[92] Y. Hirota, T. Suzuki, and Y. Bito. "The development of unusual B-cell functions in the testosterone-propionate-treated chicken." In: *Immunology* 39 (1980), pp. 29–36.

[93] J. Ivanyi. "Immunodeficiency in the chicken. II. Production of monomeric IgM following testosterone treatment or infection with Gumboro disease." In: *Immunology.* 39 (1975), pp. 1015–21.

[94] H. L, Y. Zhang, S. F. Zuo, Z. X. Lian, and N. Li. "Effects of methyltestosterone on immunity against Salmonella Pullorum in dwarf chicks." In: *Poult Sci* 88 (2009), pp. 2539–48.

[95] A. I. Al-Afaleq and A. M. Homeida. "Effects of low doses of oestradiol, testosterone and dihydrotestosterone on the immune response of broiler chicks." In: *Immunopharmacol Immunotoxicol* 20 (1998), pp. 315–27.

[96] R. D. Mittal. "Gene Editing in Clinical Practice: Where are We?" In: *Indian J Clin Biochem* 34 (2019), pp. 19–25.

[97] L. Chen, L.Tang, H. Xiang, L. Jin, Q. Li, Y. Dong, and W. Wang. "Advances in genome editing technology and its promising application in evolutionary and ecological studies." In: *Gigascience* 3 (2014), p. 24.

[98] S. H and B. Schusser. "Applications of Gene Editing in Chickens: A New Era Is on the Horizon." In: *Front Genet* 456 (2018), p. 9.

[99] H. Sang. "Transgenesis sunny-side up." In: *Nat Biotechnol* 24 (2006), pp. 955–6.

[100] P. E. Mozdziak and J. N. Petitte. "Status of transgenic chicken models for developmental biology." In: *Dev Dyn.* 229 (2004), pp. 414–21.

[101] D. W. Salter, E. J. Smith, S. H. Hughes, S. E. Wright, and L. B. Crittenden. "Transgenic chickens: insertion of retroviral genes into the chicken germ line." In: *Virology.* 157 (1987), pp. 236–40.

[102] M. J. McGrew, A. Sherman, F. M. Ellard, S. G. Lillico, H. J. Gilhooley, A. J. Kingsman, K. A. Mitrophanous, and H. Sang. "Efficient production of germline transgenic chickens using lentiviral vectors." In: *EMBO Rep* 5 (2004), pp. 728–33.

[103] A. J. Harvey, G. Speksnijder, L. R. Baugh, J. A. Morris, and R. Ivarie. "Expression of exogenous protein in the egg white of transgenic chickens." In: *Nat Biotechnol* 20 (2002), pp. 396–9.

[104] J. C. Rapp, A. J. Harvey, G. L. Speksnijder, W. Hu, and R. Ivarie. "Biologically active human interferon alpha-2b produced in the egg white of transgenic hens." In: *Transgenic Res* 12 (2003), pp. 569–75.

[105] J. Love, C. Gribbin, C. Mather, and H. Sang. "Transgenic birds by DNA microinjection." In: *Biotechnology (NY)* 12 (1994), pp. 60–3.

[106] J. N. Petitte, M. E. Clark, G. Liu, A. M. V. Gibbins, and R. J. Etches. "Production of somatic and germline chimeras in the chicken by transfer of early blastodermal cells." In: *Development* 108 (1990), pp. 185–9.

[107] B. Pain, M. E. Clark, M. Shen, H. Nakazawa, M. Sakurai, J. Samarut, and R. J. Etches. "Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities." In: *Development* 122 (1990), pp. 2339–48.

[108] K. Nishijima and S. Iijima. "Transgenic chickens." In: *Dev Growth Differ* 55 (2013), pp. 207–16.

[109] M. C. van de Lavoir, J. H. Diamond, P. A. Leighton, C. Mather-Love, B. S. Heyer, R. Bradshaw, A. Kerchner, L. T. Hooi, T. M. Gessaro, S. E. Swanberg, and R. J. E. M. E. Delany. "Germline transmission of genetically modified primordial germ cells." In: *Nature* 441 (2006), pp. 766–9.

[110] P. A. Leighton, M. C. van de Lavoir, J. H. Diamond, C. Xia, and R. J. Etches. "Genetic modification of primordial germ cells by gene trapping, gene targeting, and phiC31 integrase." In: *Mol Reprod Dev* 75 (2008), pp. 1163–75.

[111] B. Schusser, E. J. Collarini, S. M. I. Henry Yi, J. Fesler, D. Pedersen, K. C. Klasing, B. Kaspers, W. D. Harriman, R. J. E. M. C. van de Lavoir, and P. A. Leighton. "Immunoglobulin knockout chickens via efficient homologous recombination in primordial germ cells." In: *Proc Natl Acad Sci USA* 110 (2013), pp. 20170–5.

[112] L. Taylor, D. F. Carlson, S. Nandi, A. Sherman, S. C. Fahrenkrug, and M. J. McGrew. "Efficient TALEN-mediated gene targeting of chicken primordial germ cells." In: *Development* 144 (2017), pp. 928–934.

[113] N. Brookhouser, S. Raman, C. Potts, and D. A. Brafman. "May I Cut in? Gene Editing Approaches in Human Induced Pluripotent Stem Cells." In: *Cells* 6 (2017), p. 5.

[114] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier. "A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity." In: *Science* 337 (2012), pp. 816–21.

[115] E. Deltcheva, K. Chylinski, C. M. Sharma, K. Gonzales, Y. Chao, Z. A. Pirzada, M. R. Eckert, J. Vogel, and E. Charpentier. "CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III." In: *Nature* 471 (2011), pp. 602–607.

[116] B. Petersen. "Basics of genome editing technology and its application in livestock species." In: *Reprod Domest Anim* 52 (2017), pp. 4–13.

[117] L. Dimitrov, D. Pedersen, K. H. Ching, H. Yi, E. Collarini, S. Izquierdo, M. C. van de Lavoir, and P. A. Leighton. "Germline Gene Editing in Chickens by Efficient CRISPR-Mediated Homologous Recombination in Primordial Germ Cells." In: *PLoS One* 11 (2016), p. 4.

[118] Y. Song, S. Duraisamy, J. Ali, J. Kizhakkayil, V. D. Jacob, M. A. Mohammed, M. A. Eltigani, S. Amisetty, M. K. Shukla, R. J. Etches, and M.-C. van de Lavoir. "Characteristics of Long-Term Cultures of Avian Primordial Germ Cells and Gonocytes." In: *Biol Reprod* 90 (2014), pp. 1–8.

[119] H. zur Hausen. "Induction of Specific Chromosomal Aberrations by Adenovirus Type 12 in Human Embryonic Kidney Cells." In: *J Virol* 6 (1967), pp. 1174–1185.

[120] H. M. Shein, J. F. Enders, and J. D. Levinthal. "TRANSFORMATION INDUCED BY SIMIAN VIRUS 40 IN HUMAN RENAL CELL CULTURES, II. CELL-VIRUS RELATIONSHIPS." In: *Proc Natl Acad Sci USA* 48 (1962), pp. 1350–1357.

[121] H. M. Shein and J. F. Enders. "TRANSFORMATION INDUCED BY SIMIAN VIRUS 40 IN HUMAN RENAL CELL CULTURES, I. MORPHOLOGY AND GROWTH CHARACTERISTICS." In: *Proc Natl Acad Sci USA* 48 (1962), pp. 1164–1172.

[122] R. Hernandez and D. Brown. "Growth and maintenance of chick embryo fibroblasts (CEF)." In: *Curr Protoc Microbiol* (2010), pp. 4–4I.

[123] T. W. Baba, B. P. Giroir, and E. H. Humphries. "Cell lines derived from avian lymphomas exhibit two distinct phenotypes." In: *Virology* 15 (1985), pp. 139–151.

[124] D. Aumann. "Generierung genetisch modifizierter Hühnermodelle für die immunologische Forschung". July 2017. URL: http://nbn-resolving.de/urn:nbn:de:bvb:19-211576.

[125] H. Wang, M. Qi, and A. Cutler. "A simple method of preparing plant samples for PCR." In: *Nucleic Acids Res* 21 (1993), pp. 4153–4154.

[126] U. New England Biolabs Ipswich. *NEBCloner® version 1.5.0*. URL: http://nebcloner.neb.com/#!/. (accessed: 06.08.2020).

[127] L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, and F. Zhang. "Multiplex Genome Engineering Using CRISPR/Cas Systems." In: *Science* 339 (2013), pp. 819–823.

[128] A. Europe. *Zhang Lab's CRISPR Frequently Asked Questions*. URL: http://www.addgene.org/crispr/zhang/faq/. (accessed: 15.04.2021).

[129] G. Promega GmbH Mannheim. *pGL4.36[luc2P/MMTV/Hygro]*. URL: https://www.promega.de/resources/protocols/product-information-sheets/a/pgl436-vector-protocol/. (accessed: 08.06.2021).

[130] M. Clinton, D. Zhao, S. Nandi, and D. McBride. "Evidence for avian cell autonomous sex identity (CASI) and implications for the sex-determination process?" In: *Chromosome Research* 20 (2012), pp. 177–190.

[131] C. Frankl-Vilches, H. Kuhl, M. Werber, S. Klages, M. Kerick, A. Bakker, E. H. de Oliveira, C. Reusch, F. Capuano, J. Vowinckel, S. Leitner, M. Ralser, B. Timmermann, and M. Gahr. "Using the canary genome to decipher the evolution of hormone-sensitive gene regulation in seasonal singing birds." In: *Genome Biol* 16 (2015), pp. 162–9.

[132] M. Luo, Y. Yu, H. Kim, D. Kudrna, Y. Itoh, R. J. Agate, E. Melamed, and J. L. G. et al. "Utilization of a zebra finch BAC library to determine the structure of an avian androgen receptor genomic region." In: *Genomics* 87 (2006), pp. 181–90.

[133] I. E. Eder, Z. Culiga, T. Putz, C. Nessler-Menardi, G. Bartsch, and H. Klocker. "Molecular biology of the androgen receptor: from molecular understanding to the clinic." In: *Eur Urol* 40 (2001), pp. 241–251.

[134] L. L. Collins, H. Lee, Y. T. Chen, M. Chang, H. Y. Hsu, S. Yeh, and C. Chang. "The androgen receptor in spermatogenesis." In: *Cytogenet Genome Res* 103 (2003), pp. 299–301.

[135] Q. Zhou, R. Nie, G. S. Prins, P. T. K. Saunders, B. S. Katzenellenbogen, and R. A. Hess. "Localization of Androgen and Estrogen Receptors in Adult Male Mouse Reproductive Tract." In: *J Androl* 40 (2002), pp. 870–81.

[136] R. Meccariello, S. Fasano, R. Pierantoni, and G. C. (Eds.) *Modulators of hypothalamic-pituitary-gonadal axis for the control*. Frontiers Research Topics. Frontiers in Endocrinology, 2014. ISBN: 9782889193585.

[137] W. Wurst and R. K. (Eds.) *Inducible Cre Mice*. Methods in Molecular Biology. Springer Protocols, 2009. ISBN: 9781597454711.

[138] J. Rzasa, A. Sechman, H. Paczoska-Eliasiewicz, and A. Hrabia. "Effect of tamoxifen on sex steroid concentrations in chicken ovarian follicles." In: *Acta Vet Hung* 57 (2009), pp. 85–97.

[139] L. B. Menalled. "Knock-in mouse models of Huntington's disease." In: *NeuroRx* 2 (2005), pp. 465–70.

[140] T. Saito, Y. Matsuba, N. Mihira, J. Takano, P. Nilsson, S. Itohara, N.Iwata, and T. C. Saido. "Single App knock-in mouse models of Alzheimer's disease." In: *Nat Neurosci* 17 (2014), pp. 661–3.

[141] X. Wang. "Cre transgenic mouse lines." In: *Methods Mol Biol* 561 (2009), pp. 265–73.

[142] S. Li, L. Chen, X. Peng, C. Wang, B. Qin, D. Tan, C. Han, H. Yang, X. Ren, F. Liu, C. Xu, and X. Zhou. "Overview of the reporter genes and reporter mouse models." In: *Animal Model Exp Med.* 1 (2017), pp. 29–35.

[143] P. Hartig, K. Bobseine, B.Britt, M. Cardon, C. Lambright, V. Wilson, and L. Gray. "Development of two androgen receptor assays using adenoviral transduction of MMTV-luc reporter and/or hAR for endocrine screening." In: *Toxicol Sci* 66 (2002), pp. 82–90.

[144] W. Azeem, M. R. Hellem, J. R. Olsen, Y. Hua, K. Marvyin, Y. Qu, B. Lin, X. Ke, A. M. Øyan, and K. Kalland. "An androgen response element driven reporter assay for the detection of androgen receptor activity in prostate cells." In: *PLoS One* 12 (2017), p. 6.

[145] H. Makkonen, T. Jääskeläinen, M. M. Rytinki, and J. J. Palvimo. "Analysis of androgen receptor activity by reporter gene assays." In: *Methods Mol Biol.* 776 (2011), pp. 71–80.

[146] C. Campana, V. Pezzi, and W. E. Rainey. "Cell-based assays for screening androgen receptor ligands." In: *Semin Reprod Med* 33 (2015), pp. 225–34.

[147] X. Huang, X. Cang, and J. Liu. "Molecular mechanism of Bisphenol A on androgen receptor antagonism." In: *Toxicol In Vitro* 61 (2019).

[148] V. Georget, J. M. Lobaccaro, B. Terouanne, P. Mangeat, J. C. Nicolas, and C. Sultan. "Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor." In: *Mol Cell Endocrinol* 129 (1997), pp. 17–26.

[149] W. R. Strohl. "Fusion Proteins for Half-Life Extension of Biologics as a Strategy to Make Biobetters." In: *BioDrugs* 4 (2015), pp. 215–239.

[150] H. Nakauchi, K. Matsuda, I. Ochiai, A. Kawauchi, Y. Mizutani, T. Miki, and M. Kawata. "A differential ligand-mediated response of green fluorescent protein-tagged androgen receptor in living prostate cancer and non-prostate cancer cell lines." In: *J Histochem Cytochem* 55 (2007), pp. 535–44.

[151] V. Georget, B. Térouanne, S. Lumbroso, J. C. Nicolas, and C. Sultan. "Trafficking of androgen receptor mutants fused to green fluorescent protein: a new investigation of partial androgen insensitivity syndrome." In: *J Clin Endocrinol Metab* 83 (1998), pp. 3597–603.

[152] S. Okamoto, Y. Amaishi, I.Maki, T. Enoki, and J. Mineno. "Highly efficient genome editing for single-base substitutions using optimized ssODNs with Cas9-RNPs." In: *Sci Rep* 18 (2019), p. 9.

[153] T. Levi, A. Sloutskin, R. Kalifa, J.-G. T, and O. Gerlitz. "Efficient In Vivo Introduction of Point Mutations Using ssODN and a Co-CRISPR Approach." In: *Biol Proced Online* 22 (2020), p. 14.

[154] A. Kagita, M. S. Y. Lung, H. Xu, Y. Kita, N. Sasakawa, T.Iguchi, M. Ono, X. Wang, P. Gee, and A. Hotta. "Efficient ssODN-Mediated Targeting by Avoiding Cellular Inhibitory RNAs through Precomplexed CRISPR-Cas9/sgRNA Ribonucleoprotein." In: *Stem Cell Reports* 16 (2021), pp. 985–996.

[155] K. Yoshimi, Y.Kunihiro, T. Kaneko, H. Nagahora, B. Voigt, and T. Mashimo. "ssODN-mediated knock-in with CRISPR-Cas for large genomic regions in zygotes." In: *Nat. Commun.* 7 (2016), pp. 1–7.

[156] J. L. Schmid-Burgk, K. Höning, T. S. Ebert, and V. Hornunga. "CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism." In: *Nat Commun* 7 (2016), p. 12.

[157] J. F. Glenn. "Testicular feminization syndrome current clinical considerations." In: *Urology* 7 (1976), pp. 569–577.

[158] R. Souhail, S. Amine, A. Nadia, K. Tarik, E. K. Khalid, and K. A. I. A. Ahmed. "Complete androgen insensitivity syndrome or testicular feminization: review of literature based on a case report." In: *an Afr Med J* 25 (2016).

[159] J. M. Whitsett, E. W. Irvin, F. W. Edens, and J. P. Thaxton. "Demasculinization of male japanese quail by prenatal estrogen treatment." In: *Horm Behav* 8 (1977), pp. 254–63.

[160] J. Balthazart and M. Schumacher. "Estradiol contributes to the postnatal demasculinization of female Japanese quail (Coturnix coturnix japonica)." In: *Horm Behav* 18 (1984), pp. 287–97.

[161] M. J. H. Ratcliffe. "Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development." In: *Dev Comp Immunol* 30 (2006), pp. 101–18.

[162] B. Glick. "Experimental Modification of the Growth of the Bursa of Fabricius." In: *Poult Sci* 36 (1957), pp. 18–23.

[163] Y. Mase and T. Oishi. "Effects of castration and testosterone treatment on the development and involution of the bursa of fabricius and the thymus in the Japanese quail." In: *Gen Comp Endocrinol* 84 (1991), pp. 426–33.

[164] T. Ylikomi and P. Tuohimaa. "Sex steroid sensitivity of developing bursa of Fabricius." In: *Int J Dev Biol* 33 (1989), pp. 135–40.

[165] M. L. (Ed.) *Steroid Hormones and their Effect on the Immune System*. Encyclopedic Reference of Immunotoxicology. Springer, Berlin, Heidelberg, 2005. ISBN: 9783540441724.

[166] T. P. Arstila and O. Lassila. "Androgen-induced expression of the peripheral blood gamma delta T cell population in the chicken." In: *J Immunol.* 151 (1993), pp. 6627–6633.

[167] M. M. Mashaly. "Effect of Caponization on Cell-Mediated Immunity of Immature Cockerels." In: *Poultry Science* 63 (1983), pp. 369–372.

[168] G. Leitner, E. D. Heller, and A. Friedman. "Sex-related differences in immune response and survival rate of broiler chickens." In: *Vet Immunol Immunopathol* 21 (1989), pp. 249–60.

[169] S. L. Klein and K. L. Flanagan. "Sex differences in immune responses." In: *Nat Rev Immunol* 16 (2016), pp. 626–638.

[170] F. Angum, T. Khan, J. Kaler, L. Siddiqui, and A. Hussain. "The Prevalence of Autoimmune Disorders in Women: A Narrative Review." In: *Cureus* 12 (2020), p. 5.