Characterization of novel mouse monoclonal antibody against avian primordial germ cells

M.Sc. thesis

Ecology, Evolution- and Conservation Biology specialization

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Abbreviations

1. BF	Bursa of Fabricius
2. Blimp1/Prdm1	B-lymphocyte-induced maturation protein-1
3. Bmp4	Bone morphogenetic protein 4
4. BSA	Bovine serum albumin
5. CXCL12	C-X-C motif chemokine ligand 12
6. CXCR4	C-X-C chemokine receptor type 4
7. CVH	Chicken Vasa homolog
8. DAPI	4,6-diamidino-2-phenylindole dihydrochloride
9. DAZL	Deleted azoospermia-like gene
10. DMSO	Dimethyl sulfoxide
11. Dpc	Days post coitum
12. EGK	Eyal-Giladi-Kochav developmental stage
13. EMA-1	Embryonic mouse antigen-1
14. HH	Hamburger and Hamilton staging system
15. NTR	Nitroreductase
16. PALS	Periarteriolar lymphoid sheath
17. PAS	Periodic acid-Schiff staining
18. PBS	Phosphate-buffered saline
19. PELS	Periellipsoidal lymphatic sheath
20. PFA	Paraformaldehyde
21. PGC(s)	Primordial germ cell(s)
22. Prdm14	PR domain containing 14 gene
23. Tfap2c	Transcription factor AP-2 gamma
24. Tnap	Tissue nonspecific alkaline phosphatase
25. SSEA-1	Stage-specific embryonic antigen-1
26. VASA	DEAD-box RNA helicase

Introduction

In recent decades, stem cell research has experienced significant growth by integrating various biomedical disciplines such as histology, *in vitro* technology, cell biology, and genetics. Its growth is due to the increasing knowledge about stem cells and their critical roles in various biological processes (Zakrzewski et al., 2019). In the medical field, human stem cell research has established innovative techniques to better understand diseases and develop novel treatments (Nikolic et al., 2016). In biology, it has provided insights into how organs form during embryogenesis, thereby deepening our understanding of developmental biology and the developmental dynamics of stem cells. From an economic and agricultural perspective, stem cell research holds substantial promise. Cutting-edge techniques now enable the preservation of genetic material in avian species such as chickens, which are integral to global food systems and agricultural sustainability. Furthermore, these advances support the conservation of rare and endangered bird species, contributing to biodiversity preservation and promoting sustainable breeding practices in response to rising global demands.

Primordial germ cells (PGCs; precursor germ cells or gonocytes) are the precursors to gametes (Nikolic et al., 2016), and play a crucial role in genetic preservation and biodiversity efforts. However, research focusing on this type of stem cell is often hindered by the limitations of existing molecular markers (Kim and Han, 2018). To overcome this limitation, our laboratory developed a novel mouse monoclonal antibody (clone: 30B6), initially designed to identify B-cells in the bursa of Fabricius.

In my MSc thesis, I characterized this novel monoclonal antibody across a wide range of species, including avian, mammalian, reptile species, and human embryonic and adult tissues. We discovered that the 30B6 antibody not only labels early B-cells but also selectively recognizes PGCs during early avian embryogenesis. This PGC specificity offers a new tool for more precise identification and study of PGCs across various stages of development. This research not only enhances our understanding of PGCs but also provides a valuable tool for investigating molecular similarities between hematopoietic stem cells and PGCs.

Literature review

1. Primordial germ cells

The fundamental purpose of all living organisms is reproduction, ensuring the transmission of genetic information to the next generation (D'Costa and Petitte, 1999). In vertebrates, this process is mediated by germ cells. Primordial germ cells (PGCs) are unipotent cells that are formed before gastrulation and fate to produce the precursors to gametes, including oocytes and spermatozoas. Morphologically, PGCs are characterized by their large, round shape (10–20 μm) and an eccentrically positioned nucleus (Fig. 1 A) (Swift, 1914; Wentworth et al., 1989). In avian species, such as chickens, PGCs contain abundant cytoplasmic glycogen and yolk granules (Meyer, 1964). These intracellular structures are exclusively characteristic for germline cells and are not detectable in somatic cells (Wentworth et al., 1989). In vertebrate embryos, PGCs exhibit a distinctive ability to migrate and colonize the genital ridges, where interacting with the developing stroma cells of the gonad in order to continue their maturation (D'Costa and Petitte, 1999; Nakamura et al., 2013; Tagami et al., 2017).

The study of PGCs dates to Waldeyer's initial identification in 1870. A decade later, researchers proposed that PGCs originate during the early stages of embryogenesis (Kim and Han, 2018). In the early 20th century, Swift provided further evidence in birds that these cells localize along the anterior margin of the translucent central area of the blastodisc (called area pellucida), forming the germinal crescent (Swift, 1914; Kim and Han, 2018). Next, it demonstrated that PGCs emerge from the presumptive anterior pole of the embryo during the primitive streak stage before migrating to the genital epithelium (Swift, 1914; Firket, 1920). However early hypotheses suggested a hypoblast origin of the precursor germ cells (Swift, 1914), subsequent studies confirmed their derivation from the epiblast cells (Eyal-Giladi et al., 1981). Today, PGCs remain a crucial research focus due to their applications in regenerative medicine and stem cell biology (Nikolic et al., 2016).

2. Determination of PGCs

In many invertebrate and vertebrate species, precursor germ cells typically originate from extragonadal tissues and later colonize the developing gonads. Two primary models explain the PGC specification: the preformation model and the epigenesis (induction) model (Fig. 1 B) (Tagami et al., 2017; Hansen and Pelegri, 2021; Mathan et al., 2023).

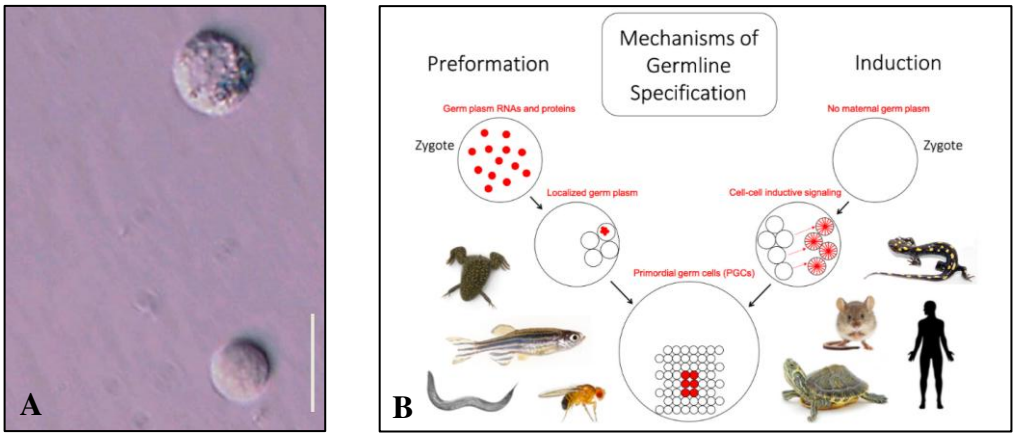

Figure 1. (A) *In vitro* morphological appearance of the chicken PGC (Tagami et al., 2017) and (B) schematic representation of two models of PGCs determination (Hansen and Pelegri, 2021).

According to the preformation model, PGC fate is determined by maternally inherited cytoplasmic structures called germ plasm (or germ granules), which contain mRNAs, small RNAs and proteins. These components become activated in the oocyte cytoplasm during or shortly after fertilization and accumulate at a certain site of the cytoplasm in order to pack into cells, ensuring germline commitment. This mechanism is observed in species such as nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), and African clawed frog (*Xenopus laevis*) (Tagami et al., 2017; Kim and Han, 2018; Mathan et al., 2023).

In contrast, the epigenesis model operates without germ plasm and relies on inductive signals from surrounding somatic cells during gastrulation (Nakamura et al., 2013; Tagami et al., 2017). This process is observed in species such as mouse (*Mus musculus*) and amphibian axolotl (*Ambystoma mexicanum*), where no germ plasm is present within the embryo (Tagami et al., 2017; Mathan et al., 2023). Birds support the epigenesis model, which will be elaborated upon in the following sections.

2.1. Mammalian PGC determination

The mouse (*Mus musculus*) is the most extensively studied mammalian model organism for testing the epigenesis model. Scientific investigation has revealed that PGC specification is regulated by inductive signals rather than inherited germ plasm. Early in embryogenesis, bone morphogenetic protein 4 (*Bmp4*) produced by the extraembryonic ectoderm and visceral endoderm initiates germline specification in proximal epiblast populations (McLaren, 2003; Nakamura et al., 2013; Tagami et al., 2017). This signalling cascade activates B-lymphocyte-induced maturation protein-1 (*Blimp1 or Prdm1*), a key transcriptional repressor that

suppresses somatic genes and promotes PGC fate. Additional factors, including PR domain containing 14 (*Prdm14*) and transcription factor AP-2 gamma (*Tfap2c*), further reinforce germline programming while inhibiting somatic differentiation (Nakamura et al., 2013; Nikolic et al., 2016; Tagami et al., 2017). *Fragilis* and *Stella* genes are essential for the development of PGCs. *Fragilis* encodes a transmembrane protein that regulates cell adhesion and signal transduction, while *Stella* produces a shuttle protein that moves between the nucleus and the cytoplasm (McLaren, 2003; Nikolic et al., 2016). Cells expressing these genes show increased *Bmp4* sensitivity, but even distal epiblast cells can take up PGC fate when exposed to these signals. The complete dependence on embryonic signals makes mice an ideal model for studying epigenesis-driven germline specification, where the cellular microenvironment alone determines fate (Tagami et al., 2017).

2.2. Avian PGC determination

In birds, early *in vitro* research supported the epigenesis model and hypoblast origin of PGCs (Karagenç et al., 1996). However, the discovery of chicken VASA homolog (DEAD-box RNA helicase, CVH, shortly VASA), a conserved germline-specific RNA-binding protein and, its asymmetric distribution during the cleavage suggests a possible preformation-like mechanism in chicken (Ichikawa and Horiuchi, 2023). A germplasm-like structure containing the VASA protein is found in the basal part of the cleavage furrow at the first cleavage stage then incorporated into 6 to 8 cells in the center of the developing blastodisc (Clawson and Domm, 1969). In avian oocytes, VASA localizes predominantly in the mitochondrial clouds, suggesting germplasm-like features similar to that in *Drosophila melanogaster*, implying maternal inheritance of germline determinants (Tsunekawa et al., 2000; Tagami et al., 2017). While these findings hint at preformation mechanism, further functional studies are needed to confirm whether avian PGC specification strictly follows this model or not.

3. Migration of PGCs

In vertebrate embryos, germline development is a complex and highly regulated process that begins with the early specification of PGCs independently of the formation of primary germ layers. Following their specification, PGCs migrate toward the developing gonads. However, the migratory mechanisms differ across species: in birds, PGCs travel through the circulatory system, whereas in mammals, they reach the gonadal ridges via active cell

migration rather than passive transport through the bloodstream (D'Costa and Petitte, 1999; Tagami et al., 2017).

3.1. Migration of mammalian PGCs

In mammals, the gonocytes (a transient population of male germ-line stem cells that are derived from PGCs and give rise to spermatogonial stem cells) undergo active migration into the gonadal ridges, with the mouse (*Mus musculus*) being the most extensively studied model organism in this context. Following fertilization, the extraembryonic cluster undergoes fragmentation within 24 hours, a process temporally associated with the downregulation of *Fragilis* (McLaren, 2003). Subsequently, PGCs within the proximal epiblast begin to express key molecular markers, including *Blimp1*, *Prdm14*, and tissue-nonspecific alkaline phosphatase (*Tnap*) (Kanamori et al., 2019). By approximately 8.5 days post coitum (dpc), the emerging endoderm undergoes morphogenesis to form the hindgut. During the initial stages of hindgut formation, PGCs may be passively transported by the collective movement of the developing endodermal layer within the forming tube (Kanamori et al., 2019). Following the formation of the hindgut, PGCs distribute in both caudal and cranial directions, initially grouped at the ventral side of the gut endodermal tube before migrating throughout its entire mesenchymal circumference (McLaren, 2003; Kanamori et al., 2019). Around 10 dpc, PGCs begin to colonize the developing gonads via the dorsal mesentery, a process mediated by chemokine receptors and dynamic cell adhesion mechanisms. This migratory phase is strongly regulated by bidirectional signalling between C-X-C motif chemokine ligand 12 (CXCL12) and its receptor, C-X-C chemokine receptor type 4 (CXCR4), which is highly expressed on migrating PGCs. Their molecular interaction not only guides PGC migration but also promotes their survival (Molyneaux et al., 2003). The CXCR4/CXCL12 signalling axis was first described in during zebrafish PGC migration (Knaut et al., 2003) and it has since been shown to play evolutionarily conserved roles in the migration of multiple cell populations, including B-cells (Nagy et al., 2020), and neural crest cells (Halasy et al., 2023). The chemokine-receptor system establishes a concentration gradient of CXCL12 that directs PGC homing to the developing gonads. Upon arrival in the gonadal environment, PGCs undergo sex-specific differentiation, committing to either the spermatogenic or oogenic lineage depending on the surrounding gonadal sex-determining signals (McLaren, 2003; Molyneaux et al., 2003; Kanamori et al., 2019).

3.2. Migration of avian PGCs

Freshly laid, fertilized egg consists of approximately 60,000 embryonic cells (Ichikawa and Horiuchi, 2023) due to the cleavage and rapid cell division occurring within oviduct and uterus of the chicken (Eyal-Giladi and Kochav, 1976). The avian zygote exhibits discoidal meroblastic cleavage: the unincubated chick blastoderm has a disc of cells corresponding to Eyal-Giladi and Kochav (EG & K) stage X (Eyal-Giladi and Kochav, 1976). In 1976, Eyal-Giladi and Kochav described the blastoderm formation of chicken supplementing the missing morphological changes until the first Hamburger and Hamilton (HH) developmental stages (Hamburger and Hamilton, 1951). They also observed the early presence of PGCs forming a cluster localized in the central region of the area pellucida at EG & K stage X (Eyal-Giladi et al., 1981), with an estimated population of 30–130 cells (Nakamura et al., 2013; Tagami et al., 2017). Subsequently, these cells migrate from the epiblast into the underlying hypoblast, where they transiently rest (Eyal-Giladi et al., 1981; Kim and Han, 2018). Next, PGCs translocate from this region, undergoing direct migration (Nakamura et al., 2007; Tagami et al., 2017; Kim and Han, 2018; Mathan et al., 2023). Initially, PGCs migration is passive, driven by the formation of the primitive streak, which facilitates their movement from the central zone of the area pellucida toward the anterior region (Mathan et al., 2023). Upon reaching the anterior region of area pellucida, PGCs adhere to fibrous bands on the basal lamina of the epiblast, resulting in the accumulation of these cells in the germinal crescent (Kim and Han, 2018). Following this migration step, PGCs of avian embryos leave the germinal crescent, they enter the circulatory system during HH9-10, with peak abundance in blood vessels at stage HH12. The circulating PGCs actively migrate towards the genital ridges between HH15 and HH18, eventually penetrating the thickened coelomic epithelium (Fig. 2). The anterior vitelline veins play a critical role in facilitating the accumulation of migration PGCs in the region (De Melo Bernardo et al., 2012).

Multiple studies have demonstrated that migrating and cultured PGCs extend pseudopodia, suggesting that amoeboid movement may be a key migratory mechanism (Kim and Han, 2018; Mathan et al., 2023). Similar to mouse and zebrafish embryos migration of the chicken PGCs is also mediated by the CXCL12 and its receptor CXCR4, expressed on the surface of migrating PGCs (Tagami et al., 2017; Kim and Han, 2018). This signalling axis is essential for directing their migration and proper localization within the developing gonads (Kim and Han, 2018). The lateral plate mesoderm shows high level of CXCL12 expression at

the time PGCs leave the circulation and conduct them toward the genital ridges (Stebler et al., 2004).

Figure 2 is from Kim and Han (2018), provides a schematic representation of PGC migration and embryonic distribution during these developmental stages.

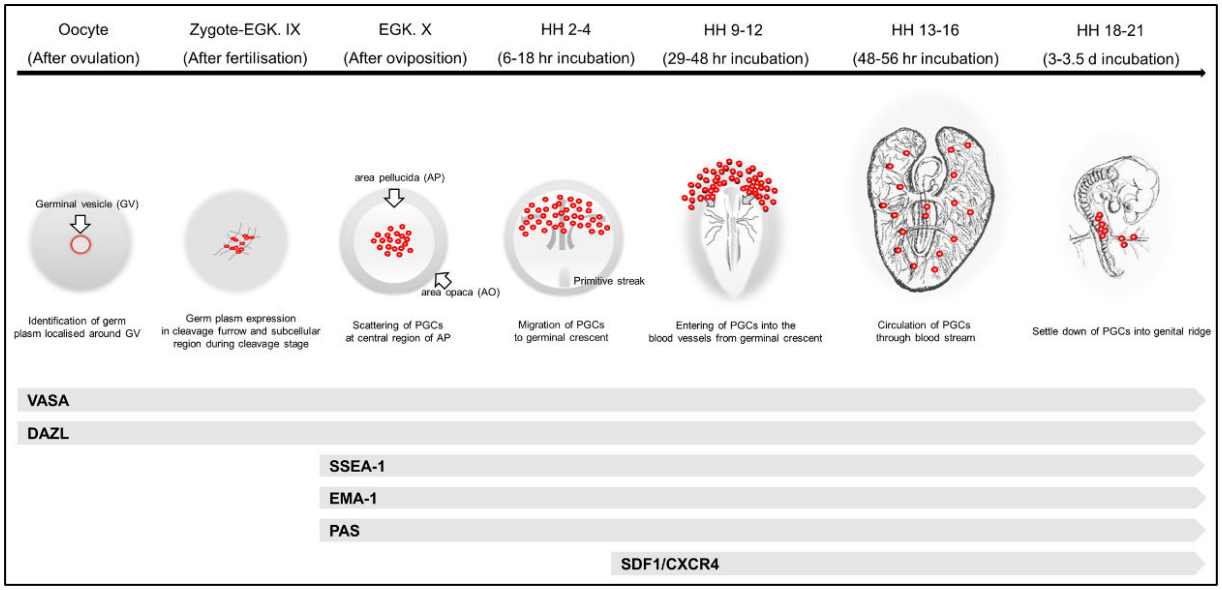

Figure 2. Migration of avian PGCs and marker activity during embryonic development (Kim and Han, 2018). The image depicts PGCs (red) undergoing sequential migration: initially localized in the germinal crescent (HH2-4), PGCs enter circulation (HH9-12), peak in bloodstream concentration (HH13-16), then extravasate (HH18-21) to colonize developing gonads, where they differentiate into gametes. Marker expression follows distinct temporal patterns: VASA and DAZL reliably identify PGCs from the earliest stages, while SSEA-1, EMA-1, and PAS positivity only become detectable after the EGK stage X. The chemokine CXCL12 serves as a late-phase marker, guiding PGC homing during final gonad colonization.

4. Identification of chicken PGCs

The precise identification of PGCs is essential for understanding their origin and developmental mechanisms. However, the number of molecular markers available for this purpose is limited, and their reliability remains suboptimal (Tsunekawa et al., 2000). Early attempts relied on histochemical staining techniques, including Meyer's 1964 study, in which he used the periodic acid-Schiff (PAS) staining method (Meyer, 1964; Kim and Han, 2018). While Meyer's technique successfully detected PGCs due to the considerable amount of cytoplasmatic glycogen, otherwise the effectiveness of PAS staining was limited identifying

PGCs in chicken embryos prior to the appearance of the primitive streak before HH4 stage (Nakamura et al., 2013).

Immunohistochemical techniques using monoclonal antibodies against primordial germ cell-specific antigens significantly advanced this field.

Two particularly important markers against chicken PGCs were developed (Fig. 2): embryonic mouse antigen-1 (EMA-1) and stage-specific embryonic antigen-1 (SSEA-1) (Karagenç et al., 1996; Tagami et al., 2017; Kim and Han, 2018; Mathan et al., 2023). The EMA-1 antibody was originally developed against mouse embryonal carcinoma cells, and it demonstrates reliable specificity for chicken PGCs from early developmental stages (HH2-4) through gonadal differentiation (Tagami et al., 2017; Kim and Han, 2018). On the other hand, SSEA-1 shows broader tissue expression limiting its utility as a definitive PGC marker despite its association with cell migration and adhesion processes (D'Costa and Petitte, 1999; Nakamura et al., 2013; Tagami et al., 2017). Importantly, neither marker reliably identifies VASA-positive germ cells during critical developmental stages HH8-19 (Fig. 2, Kim and Han, 2018).

Identifying the chicken VASA homolog (CVH) represented a major advancement in PGC research. The VASA gene is ubiquitously present in the PGCs of vertebrates. This gene encodes the VASA protein, which is critical for the specification, maintenance, migration, and differentiation of PGCs. VASA protein is an ATP-dependent RNA helicase belonging to the DEAD-box protein family (also referred to as DDX-4 or DEAD-box helicase-4) (Tsunekawa et al., 2000; Tagami et al., 2017; Lejong et al., 2020). The distribution and expression of CVH exhibit variability in chicken germ cells during the final stage of spermatogenesis. CVH is localized in both the nucleus and the cytoplasm of spermatocytes (Kito et al., 2010). This marker allows detection of PGCs from the earliest stages of embryonic cleavage through oocyte development (Kim and Han, 2018). However, recent studies have revealed unexpected CVH expression in somatic cells, questioning its status as an exclusively germline-specific marker (Lejong et al., 2020).

Another frequently used stem cell marker is the DAZL protein (Deleted in Azoospermia-Like), a member of the RNA-binding protein family. The DAZL protein is encoded by the germline-specific *Dazl* gene, which plays a critical role in gametogenesis in vertebrates (Tagami et al., 2017; Kim and Han, 2018). In case of avian species, DAZL shows characteristic nuclear and cytoplasmic localization in PGCs, and its functional importance is underscored by the azoospermia phenotype resulting from DAZL mutations (Kito et al.,

2010). However, DAZL does not provide absolute specificity for PGC identification like the other available markers.

Currently, none of the commercially available PGC markers show absolute specificity in identifying the avian PGCs. Therefore, it is recommended to apply a multiplex immunostaining approach in experimental investigations to enhance the accuracy and reliability for cell specific labelling. The continued development of novel markers and refinement of existing molecular tools remains an active area of research, as precise PGC identification is crucial for advancing our understanding of germ cell biology and its applications in reproductive technologies.

5. Applying PGCs technology to species preservation

Human activities and the emergence of new infectious diseases have an impact on both human societies and natural ecosystems. These disturbances extremely affect wild and domesticated species that are either of high ecological importance or hold significant commercial value. To mitigate biodiversity loss and preserve allelic variation in economically valuable species, cryogenic preservation of gametic material offers a practical biobanking approach, applicable within both *in situ* and *ex situ* conservation frameworks.

Birds (*Aves*) represent one of the most diverse and economically important classes within the subphylum *Vertebrata* (Tajima, 2013; Ichikawa and McGrew, 2024). According to the International Union for Conservation of Nature (IUCN), an increasing number of avian species are classified as threatened, reflecting the escalating impact of anthropogenic pressures and global environmental change on avian biodiversity (Tajima, 2013; Lázár et al., 2021). From an industrial perspective, the domestic chicken (*Gallus gallus domesticus*) is the most economically important bird species globally due to its central role in meat and egg production (Tajima, 2013; Silyukova et al., 2020; Ichikawa and McGrew, 2024). The conservation and protection of genetic diversity within both traditional (heritage) breeds and high-yield commercial lines are of critical importance (Tajima, 2013; Mathan et al., 2023). Maintaining genetic variability is essential not only for the long-term sustainability and resilience of poultry production systems but also for safeguarding the unique genetic resources found in rare or regionally adapted breeds (Tajima, 2013; Nandi et al., 2016). Furthermore, it is essential to protect the economically important species genetic resources from pandemics which can occur often in poultry production, disease like avian influenza or, infectious bursal disease (IBDV) (Nandi et al., 2016; Oláh et al., 2022).

One of the most widely known and commonly applied methods for genetic preservation is the cryopreservation of PGCs or gonadal tissues, performed at ultra-low temperatures. This technique was introduced approximately 70 years ago, primarily for the long-term storage of gametes such as sperms and oocytes (Mathan et al., 2023). The cryopreservation process begins with sample collection, where target cells or tissues are isolated. While cryopreservation has proven to be effective in many cases, subsequent research has revealed that certain steps of the process can induce cellular damages. These include irregularities in cell structure, alterations in membrane proteins and lipids, and the generation of oxidative stress, all of which may compromise cell viability and functional integrity after thawing (Mathan et al., 2023). While rooster semen cryopreservation is a well-established technique, successful fertilization requires high doses of motile sperm due to anatomical and physiological challenges (Tajima, 2013; Ichikawa and McGrew, 2024).

In contrast, traditional sperm freezing is mainly used for preserving individual genetic traits, isolating and cryopreserving PGCs at an early stage, offers a more comprehensive solution for preserving an organism's complete germline genetic material. By storing PGCs and later injecting them into host embryos, scientists can produce chimeric organisms with functional reproductive cells containing the original donor's genetic material. This method provides a powerful tool not only for maintaining genetic diversity but also for revitalising rare or commercially significant genetic lineages (Petitte, 2006; Mathan et al., 2023; Ibrahim et al., 2024).

5.1. Germline chimeras

A germline chimera is an organism having reproductive cells (sperms or oocytes) originated partly or entirely from another species' donor cells with different genetic background (Fig. 3). These types of chimeras are typically achieved by transplanting donor PGCs into a host embryo. After the host has successfully hatched than reached sexual maturity, it can produce donor-originating gametes, and the donor's genes could be passed on to the next generation. In birds, multipotent either blastodermal cells or PGCs are used to generate germline chimeras or create transgenic individuals (Tajima et al., 1993; Nakamura et al., 2010). The success of the cell transplantation and the productivity of the prospective germline chimeras depend on the proper sterilization of the host (Nakamura et al., 2010).

Elimination of the recipient PGCs could be achieved by applying busulfan treatment (Song et al., 2005; Nakamura et al., 2010). Busulfan (1,4-butanediol dimethanesulfonate) is a

potent alkylating cytostatic agent that selectively inhibits the proliferation of dividing germ cells while inducing cellular damage or apoptosis in them, thereby causing depletion of PGCs in the developing gonadal ridges. The primary objective of this technique is to clear or minimize the host's PGCs, thereby enhancing the donor PGC colonization efficiency. This approach ensures that the germline of the resulting offspring is donor-derived, enabling the production of progeny with the desired genetic background. Effectiveness of busulfan treatment is dose dependent producing a semi-sterile state in host embryos in most cases (Nakamura et al., 2010).

Scientists have explored strategies such as crossing the chimeras back with the original donor to obtain offspring with exclusively donor-derived genomes in later generations (Lázár et al., 2021). A major advantage of this approach is its ability to preserve both male and female germline components, including W chromosomes and mitochondrial DNA, which cannot be maintained through traditional sperm or embryo freezing. Nevertheless, current success rates remain low, necessitating further optimization (Lázár et al., 2021).

Transgenic animals, including those with self-eliminating PGC systems, are the latest innovative scientific methods that facilitate the development of transplanted PGCs. Recently, Chen et al., generated a gene-edited new chicken strain, (the germ-cell Specific AutonoMoUs RemovAI Induction: gSAMURAI) that eliminates its own PGCs in response to a specific drug. Applying CRISPR-Cas9 technology the chicken VASA homolog gene locus was used to drive the expression of an extra enzyme (nitroreductase, NTR) in a germ-cell specific way (Chen et al., 2023). NTR-mediated cell ablation technique is widely applied method in developmental biology, based on the ability of the nitroreductase enzyme to convert an non-toxic prodrug, the metronidazole into cytotoxic metabolite (Curado et al., 2008). Modified germ cells are tagged with a fluorescent marker (e.g., mCherry) for tracking purposes. In the gSAMURAI chicken strain the nitroreductase/metronidazole system produces a sterile germ cell niche, allowing transplanted donor PGCs to differentiate without competition. The system supports exclusive donor-derived gamete development, representing a significant advancement in precision breeding technologies using the PGC technology (Chen et al., 2023).

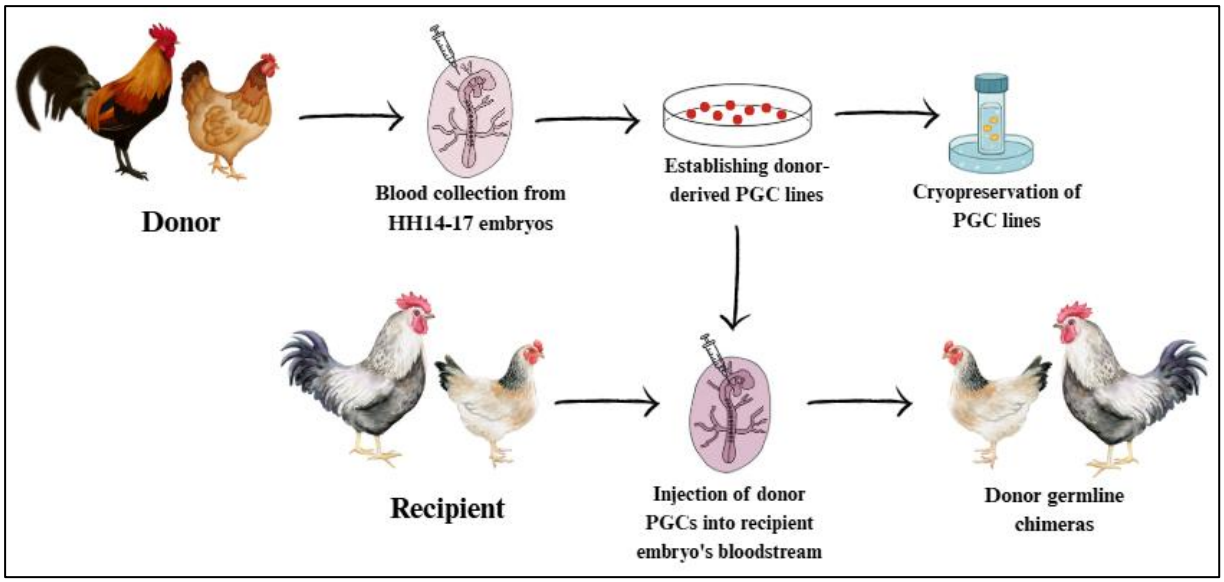

Figure 3. Generation of germline chimeras with intraembryonic injection of PGCs. The process begins with blood sample collection from the donor embryos. Established donor PGC cell cultures are subsequently injected into the host embryo's bloodstream. After hatching, PGC-derived chimeras are produced, enabling the incorporation of the donor genetic material into the germline of the recipient (the figure is the author's own work).

Aims

The Laboratory of Stem Cell and Experimental Embryology at Semmelweis University developed a novel mouse monoclonal antibody, designated 30B6. In my MSc thesis, I characterized the 30B6 reactivity across multiple species—including various avian species, one reptile species, and human specimens—as well as at different developmental stages.

The primary aims of my research were:

- To characterize the staining pattern of the 30B6 antibody in adult lymphoid and visceral organs, including the gonads.
- To investigate the spatial and temporal distribution of 30B6 during the development and migration of avian PGCs.

Materials and Methods

1. Animals

The experimental animals were White Leghorn chickens (*Gallus gallus domesticus*), and fertilized eggs obtained from commercial breeders (Prophyl-BIOVO Ltd, Hungary). The eggs were incubated at 37.5 °C in a humidified (60% relative humidity) incubator (Heka-Brutgerate, TS-7000C). The age of the embryos were determined either using the HH stage (Hamburger and Hamilton, 1951) or in case of older embryos the numbers of incubation day were utilized. Besides embryonic tissue samples, lymphoid organs and gonads were obtained from 6-8 weeks. The hatched birds were sacrificed by cervical dislocation and tissue samples were collected from at least three animals per age group. Frozen sections from adult turkey (*Meleagris gallopavo domesticus*), partridge (*Perdix perdix*), guineafowl (*Numida meleagris f. domestica*), a reptile species (*Chamaeleo calyptratus*), and human intestinal.

Avian experiments were approved by the Institutional Animal Care and Use Committee of Semmelweis University, Budapest, Hungary.

2. Histological analysis of the samples

2.1. Preparation of gelatine-embedded blocks

Tissue samples were initially fixed in 4% buffered paraformaldehyde (PFA) at room temperature for 1 hour. After fixation, the samples were washed three times for five minutes in phosphate-buffered saline (PBS, pH 7,2) and incubated in 15% D(+) sucrose (Molar Chemicals Kft., cat.no: 02200-203-190) solution in PBS at 4-°C for overnight. The following day, the sucrose solution was replaced with 7.5% gelatine (Sigma, cat.no: G2625), and 15% sucrose, and the tissues were further incubated at 37.5°C for 2 hours. Meanwhile, plastic plates were prepared by filling halfway with gelatine solution. The samples were then placed onto the solidified gelatine layer, properly oriented, and covered with a second gelatine layer. The orientation of each organ was marked at the proximal and distal ends to ensure correct orientation during cryosectioning. After embedding, the tissue blocks were cut out with scissors, placed on a piece of cardboard, and rapidly frozen in -40°C isopentane (2-methylbutane, Sigma-Aldich, cat.no: 102570052). Until sectioning, the blocks were stored at -20°C.

2.2. Preparation of liver-embedded blocks

The dissected organs were stored in 1x PBS until tissue block preparation. During embedding adult chicken, a liver slice was placed on cardboard where the extracted organs were positioned and then completely covered with thin liver pieces. The prepared liver block was frozen in liquid nitrogen vapor at -120°C. Until further use, the blocks were stored at either -20°C or -80°C.

2.3. Cryosectioning

For sectioning, I used a Shandon cryotome, set to -20°C for liver-embedded blocks and -25°C for gelatine-embedded blocks. Before sectioning, the blocks were placed in the cryostat to reach its temperature. Tissue samples were sectioned at a thickness of 10 μm and mounted on poly-L-lysine-coated slides (Tribioscience, cat.no: TBS8025-500ML). In case of liver-embedded blocks, after the sections dried at room temperature, they were fixed in 4°C acetone (Molar Chemicals Kft., cat.no: 00620-493-187) for 10 minutes.

3. Immunohistochemistry and immunofluorescent staining

3.1. Immunohistochemistry

The frozen gelatine sections were rehydrated in pre-warmed 1× PBS for 10 minutes. Afterward, the sections were placed in a humid chamber and incubated with primary antibodies (Table 1), for 1 hour at room temperature. Next, the sections were washed three times with 1× PBS for 5 minutes each and this step was applied between every incubation period. Biotinylated secondary antibodies (Table 2) were selected based on the isotype and host specificity of the primary antibody and diluted 1:200 in 1× PBS containing bovine serum albumin (BSA). The sections were incubated with secondary antibodies for 45 minutes at room temperature. To detect antigen-antibody binding, a colour reaction catalysed by peroxidase enzyme was utilized. Blocking of the endogen-peroxidase activity was performed using 3% hydrogen peroxide (Sigma Aldrich, cat.no: H1009) solution in 1x PBS (10 minutes at room temperature). Before the application of avidin-biotin peroxidase complex (ABC, Vectastain Elite ABC kit, Vector Laboratories, cat.no: PK-6100) the sections were rinsed in 1x PBS (3x 5 minutes). The ABC was prepared in a dilution recommended by the company (1x PBS at a 1:100 ratio) and applied to the sections for 30 minutes at room temperature. In parallel, a 4-chloro-1-naphthol (Sigma, cat.no: C8890) solution (96% dissolved in ethanol)

was prepared by diluting 50 µl of the reagent in 50 ml of 1x PBS. The solution was mixed in the dark on a magnetic stirrer for 30 minutes, then filtered, and 250 µl of 3% hydrogen peroxide was added. After the ABC incubation, the sections were washed three times in 1x PBS for 5 minutes each and then incubated in the 4-chloro-1-naphthol solution in the dark for 30 minutes. Peroxidase enzyme activity, bound to the antibody-ABC complex, was visualized through a blue colour reaction. Finally, the slides were covered with water-based mounting media (Mowiol) and stored at 4°C.

3.2. Single immunofluorescence staining

Following the rehydration process (in prewarmed 1x PBS for 10 minutes) the frozen tissues were incubated with primary antibodies (Table 1) for 1 hour at room temperature and washed in PBS (3x5 minutes). Alexa-conjugated fluorescent secondary antibodies (Table 2) diluted 1:200 in 1× PBS. The sections were incubated with secondary antibodies for 45 minutes at room temperature in a humid chamber. Cell nuclei were stained with DAPI (4,6-diamidino-2-phenylindole dihydrochloride, Invitrogen, cat.no: D1306) for 10 minutes, followed by washes in 1× PBS (3x5 minutes). The final step was mounting the slides with water-based mounting media and storing them at 4°C.

3.3. Double immunofluorescence staining

With double immunofluorescence staining, we examine the co-expression of different molecules. For this, we use two primary antibodies of different isotypes and/or species (Table 1.), along with corresponding fluorochrome conjugated secondary antibodies (Table 2.).

For double immunofluorescence sections were incubated with primary antibody (Table 1) for 1 hour at room temperature which was followed by washing with PBS (1x PBS, 3x5 minutes). Then the first Alexa-conjugated secondary antibody (Table 2) was applied to the sections for 45 minutes in a dark chamber. Nevertheless, the remaining steps of the staining, involving the washing procedures, were executed in dark, protecting the fluorescent dyes from light. In the next steps, the second primary antibody was applied for 1 hour, followed by another round of washing (1x PBS, 3x5 minutes). The second Alexa-conjugated secondary antibody was chosen to have fluorescence different from that of the previously used secondary antibody and it was on the sections for 45 minutes. Finally, DAPI was applied to staining the cell nuclei. At the end of the staining, the sections were washed (1x PBS, 3x5

minutes) then, covered with water-based mounting media, and until the microscopic observation thew were stored at 4°C.

3.4. Whole-mount immunostaining

Whole embryo staining was conducted during the early developmental stages of chicken embryos, specifically between HH4-HH12 stages. Embryos were carefully extracted using forceps and an embryo scoop, then transferred to a Petri dish containing 1× PBS. Fixation was performed with 4% PFA for 3 hours, followed by three washes of 20 minutes each with 1x PBS on an orbital shaker. Then the embryos were incubated with the primary antibody (clone: 30B6) for 3 hours on the laboratory shaker which was followed by washing in 1x PBS (3x20 minutes, on shaker). The Alexa-conjugated secondary antibody was applied to the whole embryo for 3 hours. After the last wash (in 1x PBS, 3x20 minutes) the whole embryos were covered with water-based mounting media and stored at 4°C.

Table 1. Primary antibodies.

Primary antibody	Source (host species)	Isotype	Dilution	Antigen specificity	Supplier/Origin
SSEA-1	mouse	IgM	1:100	anti-CD15	DSHB
EMA-1	mouse	IgM	1:100	against glycolipids of cell surface glycoproteins specific to PGCs	DSHB
anti-CXCR4 (clone 9D9)	mouse	IgG_{2a}	supernatant	CXCR4 receptor	Dr. Sonja Hartle, München University
DAZL	rabbit	IgG (H+L)	1:100	endogenous levels of total DAZL protein in germ cells	DSHB
P63	rabbit	IgG	1:00	p63 gene coded protein in the PGCs	DSHB
chB6	mouse	IgG1	supernatant	Bu1a-Bu1b on B cells	Dr. Nándor Nagy, Semmelweis University (Igyártó et al., 2008)

Grl1	mouse	IgG (H+L)	1:200	recognise granulocytes, activated thrombocytes, acinar cells of pancreas	DSHB
CD44	mouse	IgG_1	1:200	labels macrophages	DSHB
CD45	mouse	IgG_{2A}	1:200	common leukocyte marker	Prionics Co.

Table 2. Secondary antibodies.

Secondary antibody	Emitting wavelength of the fluorochrome	Organisms of origin	Supplier (catalogue number)
anti-mouse IgG (H+L)	488 nm	Donkey	Invitrogen (A21202)
anti-mouse IgM	488 nm	Goat	Invitrogen (A21042)
anti-rabbit IgG	594 nm	Donkey	Invitrogen (A21207)
anti-mouse IgG (H+L)	594 nm	Donkey	Invitrogen (A21203)
anti-mouse IgM	594 nm	Goat	Invitrogen (A21044)
Biotinylated anti-mouse IgM	-	Goat	Vector Laboratories (8A-9200)

4. Used solutions used for cytochemistry

1 liter of 10x PBS: 80 g NaCl, 0,2 g KCl, 14,4 g $Na_2HPO_4 \cdot 2\ H_2O$, 0,2 g KH_2PO_4 and distilled water.

1x PBS: 1/10 of 10x PBS mixed with 9 parts of distilled water

15 m/V% sucrose: 15 g D(+) sucrose (Molar Chemicals Kft., cat.no: 02200-203-190) dissolved in 100 ml 1x PBS.

7,5 m/V% Gelatine: 75 g D(+) sucrose (Molar Chemicals Kft., cat.no: 02200-203-190) and 37.5 g gelatine (Sigma, cat.no: G2625) dissolved in 500 ml warm 1x PBS on a magnetic mixer.

4 % Buffered Paraformaldehyde (PFA, pH = 7,4): 20 g of paraformaldehyde (Merck, cat.no: 1.04005.1000) dissolved in 400 ml 80oC distilled water on a magnetic mixer. 100 µl 1N NaOH was added to help dissolve any remaining PFA precipitate. The solution was filtered

through filter paper and 50 ml of 10x PBS was added, then completed with distilled water to the final volume of 500 ml.

1 % PBS-BSA: 1 g of BSA (Sigma, cat.no: A9647-50G) dissolved in 100 ml PBS. After filtration 0,1 % Na-azide was added (NaN_3).

Water-based mounting media (Mowiol): 5 ml of glycerol (Reanal MSZ 9529, 87% glycerin) and 2.4 g of Mowiol (Sigma-Aldrich, cat.no: 81381-250G) were mixed in a 15 ml Falcon tube. Then, 6 ml of distilled water was added, and the mixture was left to rest for 2 hours at room temperature. After that, 12 ml of 0.2 M Tris-HCl buffer (pH 8.5) was added, and the solution was incubated in a 50°C water bath for 1 hour. Finally, the mixture was centrifuged using a Jonan Centrifuge for 15 minutes.

4-chloro-1-naphtol solution: 500 mg of chloronaphthol and 2 ml of absolute ethanol were mixed and stored at 4°C.

5. Evaluation of the samples

The evaluation of the sections was carried out using fluorescence, and confocal microscopy techniques. The fluorescence microscopic images were captured using an Olympus DP74 camera connected to a Nikon Eclipse E800 microscope, with the assistance of CellSens software. The confocal microscope images were obtained using a Zeiss LSM 710 scanning confocal microscope and processed with ZEISS ZEN Imaging Software. Digital image processing was carried out using ImageJ and Adobe Photoshop CC 2019 software.

Results

1. Expression pattern of the 30B6 antigen across vertebrate species.

The 30B6 monoclonal antibody was developed in the Laboratory of Stem Cell and Experimental Embryology at Semmelweis University. Monoclonal antibodies were generated against a guinea fowl bursal cell suspension using the standard hybridoma technique (Nagy et al., 2001). In brief, Balb/c mice were immunized with a cell suspension derived from the bursa of Fabricius (BF) of guinea fowl (*Numida meleagris*). After immunization, B-cells were fused with myeloma cells, and the resulting hybridomas were screened and selected for antibody production. The 30B6 antibody was then purified from the culture supernatant of these hybridomas.

In this study, as a first step the tissue distribution of the antigen recognized by the 30B6 antibody was examined in various primary and secondary lymphoid organs of adult guinea fowl using immunohistochemistry. Strong immunoreactivity was detected in the BF, a primary lymphoid organ responsible for B-cell development and maturation in birds. Expression of the 30B6 antigen was specifically localized to the medullary region of the bursal follicles (Fig. 4 A). These follicles are primarily composed of B-cells distributed between the cortical and medullary zones. To confirm the identity of these cells, a B-cell–specific antibody, chB6 (targeting the Bu-1 antigen, a death receptor expressed predominantly on avian B-cells), was used (Fig. 4 A') (Igyártó et al., 2008). Double immunofluorescence staining showed that B-cells within the medulla also expressed the 30B6 antigen (Fig. 4 A"). In another primary lymphoid organ, the thymus (responsible for T cell development), 30B6 exhibited sporadic immunoreactivity in the medullary region (Fig. 4 B), consistent with the presence of a minor chB6+ B-cell population in this predominantly T-cell environment (Fig. 4 B'–B").

Among secondary lymphoid organs, 30B6 expression was especially evident in B-cell–rich regions. As in mammals, the avian spleen is organized into white pulp and red pulp. The white pulp includes periellipsoidal lymphatic sheaths (PELS), predominantly containing B-cells, and periarteriolar lymphatic sheaths (PALS), which are T-cell–rich areas. Immunofluorescence staining with 30B6 revealed strong immunoreactivity in the PELS and germinal centres (Fig. 4 C), closely matching the distribution pattern of the chB6 antibody (Fig. 4 C'–C"). The caecal tonsil, the largest gut-associated secondary lymphoid organ in birds, is located anti-mesenterically at the base of the caecum. Like in mammals, its

submucosal layer contains B-cell–dependent germinal centres and interfollicular T-cell regions. The 30B6 antibody weakly labelled the germinal centres (Fig. 4 D), again mirroring the distribution of the chB6 marker (Fig. 4 D'–D"). Additionally, strong and uniform 30B6 immunoreactivity was observed along the intestinal epithelium lining the caecum (Fig. 4 D, D").

To further assess the antibody's specificity, cross-species reactivity was examined in a range of vertebrate tissues. Immunohistochemical analysis of the chicken (*Gallus gallus domesticus*) BF revealed a staining pattern similar to that observed in guinea fowl, with strong 30B6 immunoreactivity localized to medullary B-cell populations within the follicles (Fig. 5 A–A"').

Additional immunostaining included tissues from Hungarian partridge (*Perdix perdix*) (Fig. 5 B), chameleon (*Chamaeleo calyptratus*) (Fig. 5 C–D), and postnatal human intestinal sections (Fig. 5 E). In these species, 30B6 specifically labelled mucus-producing goblet cells within the intestinal epithelium, rather than immune cell populations. These distinct binding patterns suggest that the 30B6 antibody recognizes a conserved epitope shared by: 1) a developmentally regulated surface glycoprotein on avian B-cells, and 2) secretory mucin glycoproteins in the intestinal mucosa of various vertebrates. Further biochemical characterization is necessary to precisely identify the molecular target of the 30B6 antibody.

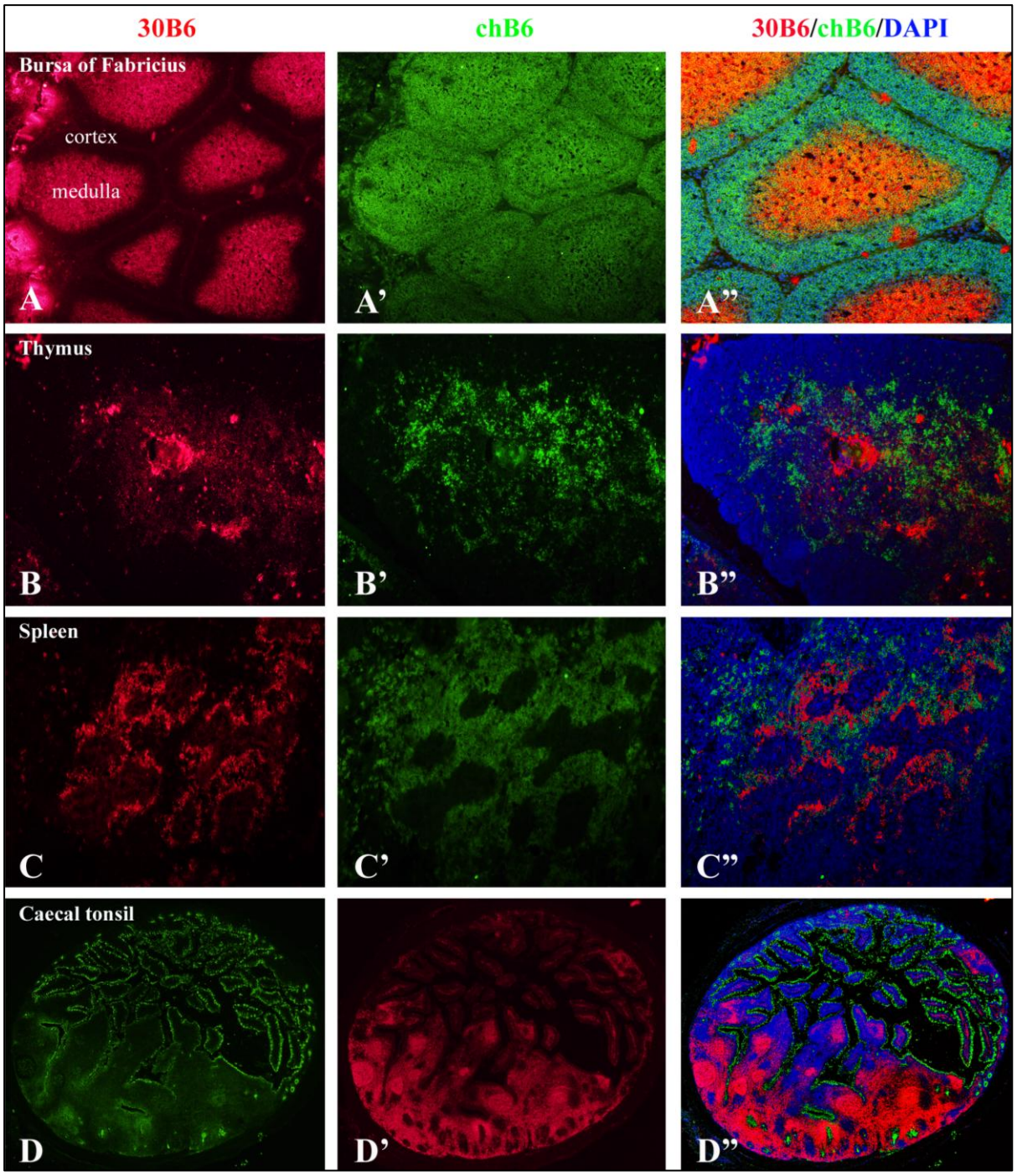

Figure 4. Localization of 30B6-immunopositivity in the lymphoid organs of adult guinea fowl (*Numida meleagris*). Immunofluorescence staining of BF (A–A"), thymus (B–B"), spleen (C–C"), and caecal tonsil (D–D") from 6-week-old birds. In the bursa, 30B6 immunoreactivity is specific to the follicular medulla (A), co-localizing with chB6+ B-cells (A'–A"). In the thymus, medullary regions show disseminated 30B6 immunoreactivity (B), overlapping with a minor population of chB6+ B-cells (B'–B"). In secondary lymphoid tissues (C–D"), 30B6 weakly labels B-cell–dependent areas, including PELS in the spleen and germinal centres in the caecal tonsil.

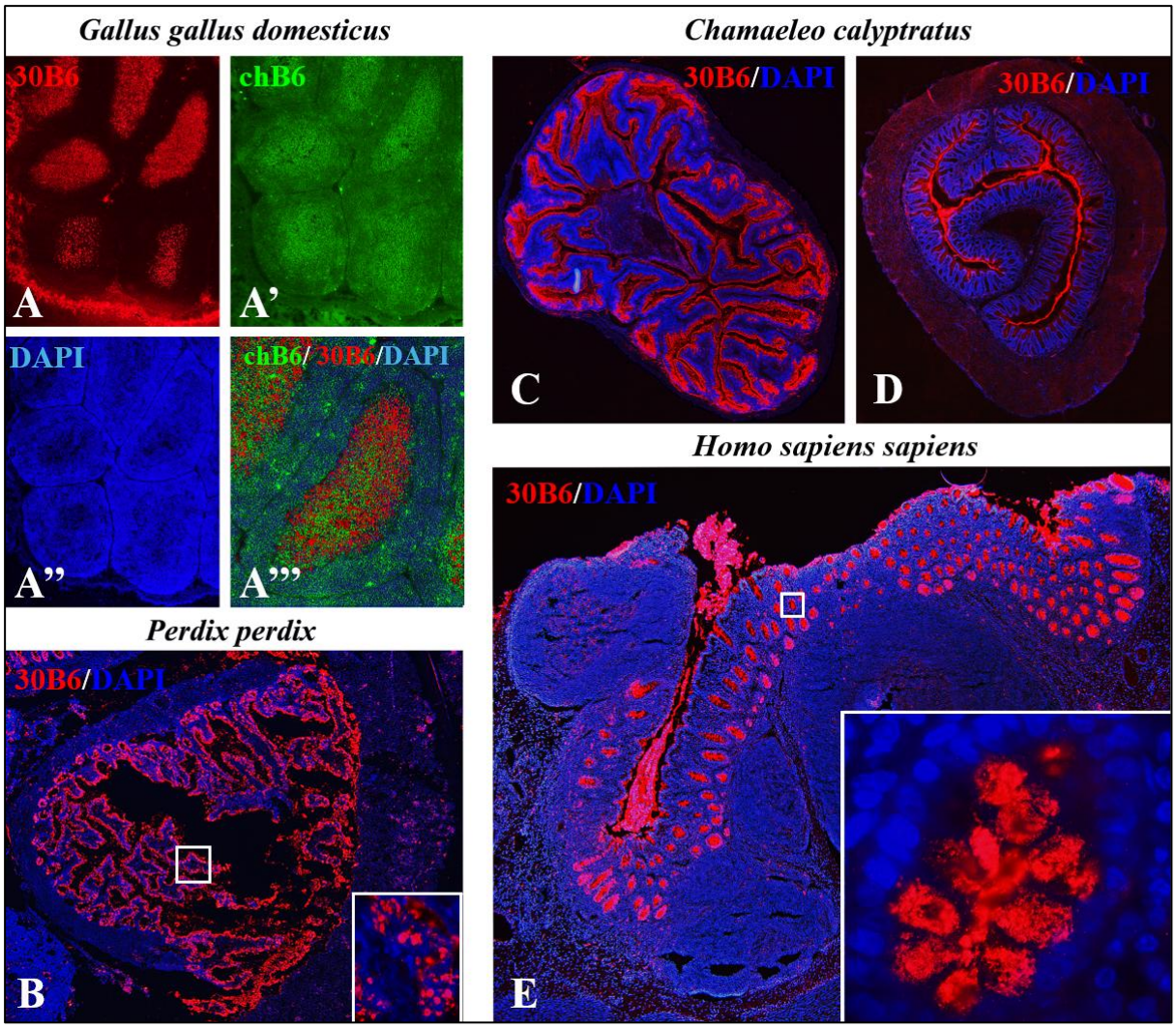

Figure 5. Cross-reactivity of 30B6 in different vertebrate species. In addition to B-cell labelling in the chicken bursa (A–A'''), intense 30B6 immunoreactivity is observed in mucin-producing goblet cells and the mucosal layer of the intestinal epithelium in the Hungarian partridge colon (B), chameleon small intestine (C) and colon (D), and human large intestinal tissue (enlarged photo about the goblet cells rich Lieberkühn-crypts of colon) (E).

2. Detection of the 30B6 antigen in the embryonic chicken bursa of Fabricius.

To comprehensively evaluate the specificity of the 30B6 antibody, we have examined embryonic BF tissues from 14-day-old (Fig. 6 A-G) and 18-day-old (Fig. 6 H-K) chicken embryos. The characteristic follicular structure formation of the bursa is preceded by hematopoietic cell colonization, which occurs in three distinct waves between embryonic days 10 and 15 (Le Douarin et al., 1975; Nagy et al., 2004). These incoming cells express the common leukocyte antigen, CD45 (Nagy et al., 2004; Szőcs et al., 2024).

The first wave of blood-borne cells includes Grl1+/Grl2+ granulocytes, which remain in the mesenchyme of the bursal folds and never colonize the follicular buds (Quesada and Agulleiro, 1984; Nagy et al., 2020). During the second wave, EIV-E12+ lymphoid inducer cells migrate into the bursal epithelium, initiating follicular bud formation. This is followed by the entry of CD45+/74.3+ precursors of bursal secretory dendritic cells (Nagy et al., 2004; Dóra et al., 2017; Szőcs et al., 2024). The final wave involves CD45+/chB6+ B-cell precursors that initially spread through the mesenchyme and later colonize the dendro-epithelial follicular buds (Nagy et al., 2020; Fejszák et al., 2022). To delineate the cell populations recognized by the 30B6 antibody, double immunocytochemical staining experiments were performed using lineage-specific markers. By embryonic day 14 (E14), bursal folds lined by epithelium are well differentiated, with numerous CD45+ cells located in the developing follicular buds and central mesenchymal regions of the folds (Fig. 6 A). The distribution of 30B6+ cells partially overlapped with CD45+ cells (Fig. 6 B-C). Double immunofluorescence revealed sporadic 30B6+/CD45+ cells within the bursal mesenchyme (Fig. 6 D). Next, using an anti-CD44 antibody, which targets an adhesion molecule essential for hematopoietic and B-cell migration and survival (Corbel et al., 2000), we showed partial co-localization with 30B6 (Fig. 6 E). This pattern was similar to that observed with the chB6 antibody, which is also expressed by 30B6+ cells (Fig. 6 F). None of the 30B6+ cells in the bursa mesenchyme co-expressed the granulocyte-specific marker Grl1/Grl2 (Fig. 6 G). These findings support the hypothesis that 30B6 identifies migratory B-cell subpopulations bursal primordium. By day 18 (E18), follicular bud formation is well-developed, as confirmed by chB6 staining (Fig. 6 H, J). Some chB6+ B-cells in the follicles also expressed the 30B6 antigen (Fig. 6 H, I, K), suggesting that 30B6 preferentially marks more mature B-cell subsets, while chB6 labels both immature and mature B-cells. In addition to lymphoid cells, 30B6 antibody also showed strong immunoreactivity on the surface epithelium lining the bursa (Fig. 6 H, I, K), similar to the staining patterns in adult avian and human intestinal epithelium.

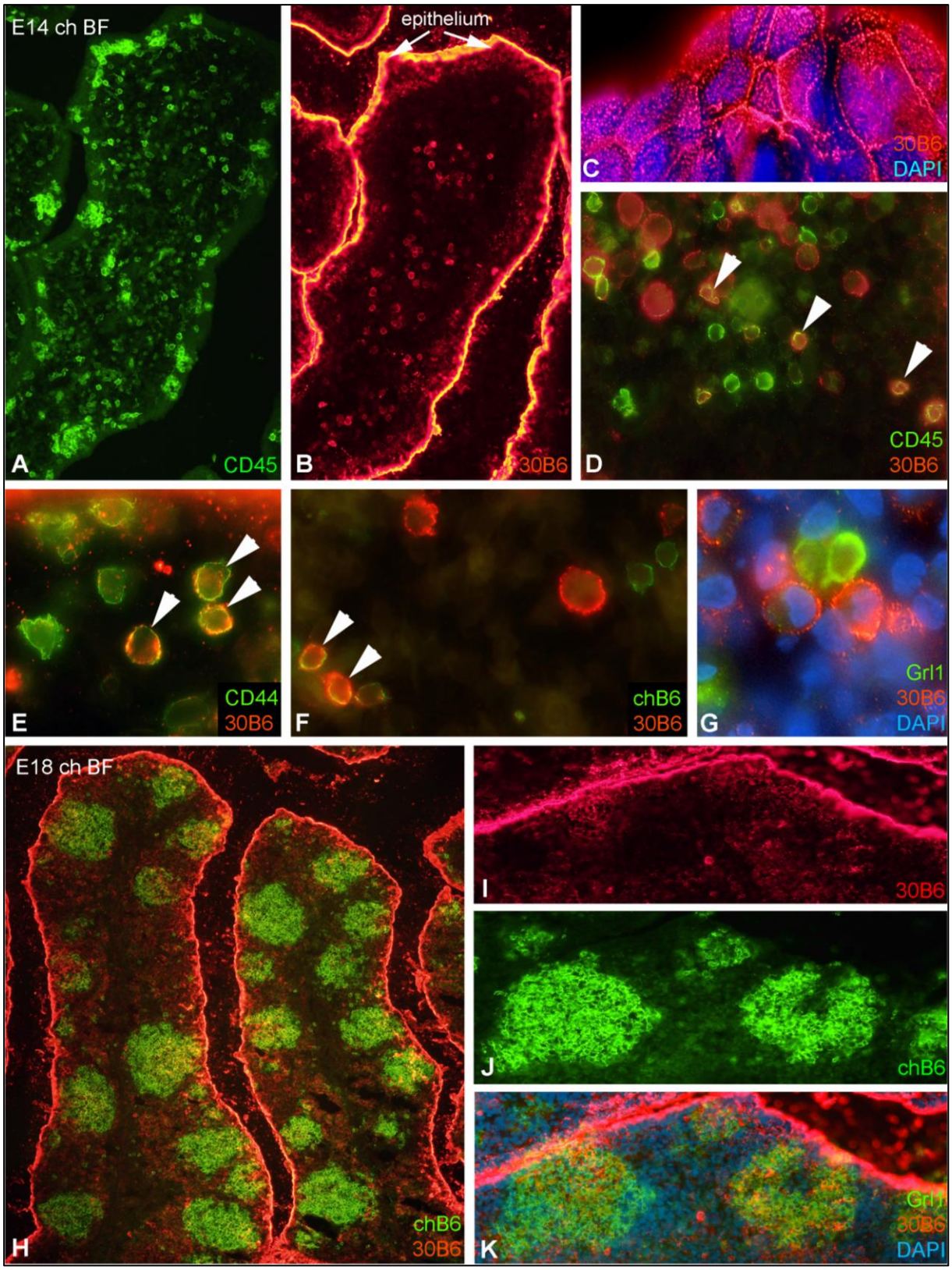

Figure 6. Detection of 30B6-immunopositive cells during chicken bursa of Fabricius development. Consecutive sections from E14 (A-G) and E18 (H-K) embryos were double-stained with 30B6 (red) and lineage-specific markers: hematopoietic (CD44, CD45, green), B-cell (chB6, green), and granulocyte (Grl1, green).

3. Temporal and spatial distribution of 30B6-positive PGCs during embryonic development.

Although originally developed as a B-cell marker, the 30B6 antibody was also found to label PGCs. To investigate the emergence and distribution of 30B6+ PGCs, we analysed chicken embryos at various developmental stages. At HH4, whole-mount immunofluorescence staining revealed large, round 30B6+ cells grouped in a crescent shape anterior to the primitive streak along the area opaca/area pellucida boundary (Fig. 7 A). Cross-sections and double staining with DAZL, a well-known PGC marker, confirmed co-localization on PGCs (Fig. 7 B-B"). Most DAZL+ cells (Fig. 7 B', B") were also 30B6+ (Fig. 7 B, B"), supporting the conclusion that 30B6 labels PGCs in the germinal crescent.

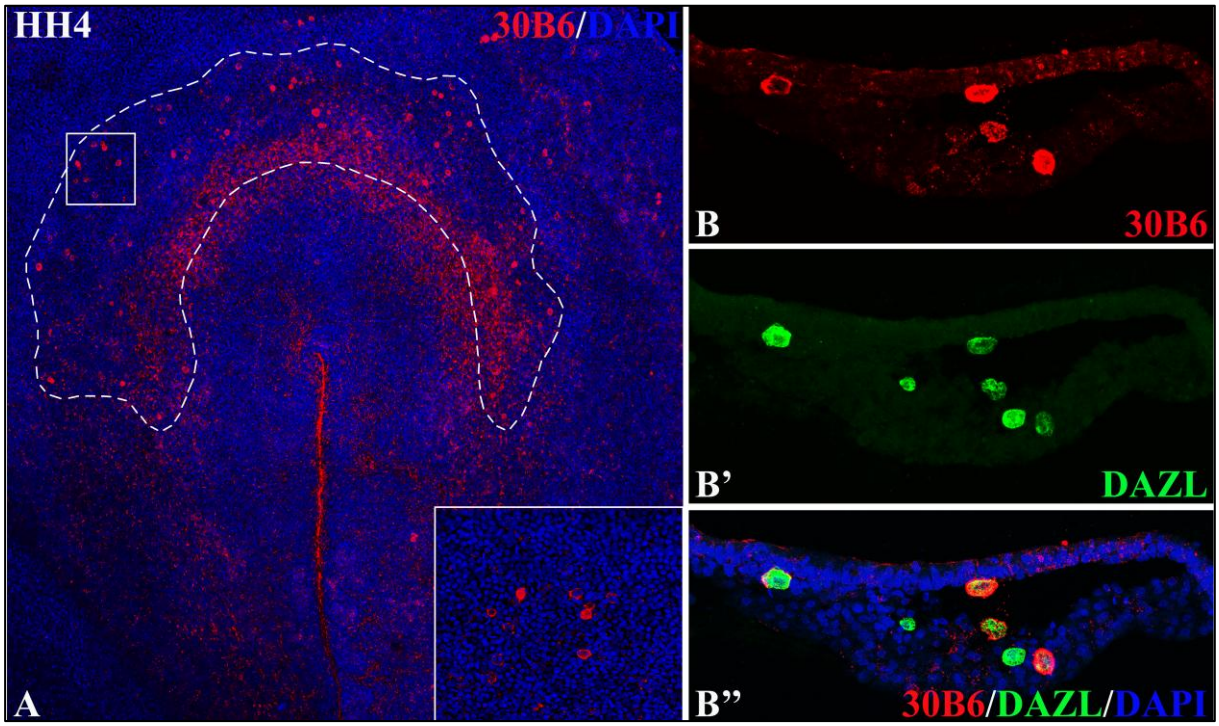

Figure 7. Detection of primordial germ cells in HH4 chicken embryos using 30B6 and DAZL antibodies. (A) Whole-mount staining with 30B6 (red); (B-B") cross-sections show partial co-localization of 30B6 (membrane) and DAZL (cytoplasm).

As development progressed, PGCs migrated from the germinal crescent through the bloodstream to the gonads. Immunofluorescence staining at E4, E6, and E8 revealed that at E4, 30B6+ cells were located ventral to the dorsal aorta at the mesonephros level (Fig. 8 A). By E6, they had exited the posterior body wall and begun colonizing the genital ridges (Fig. 8 B). At E8, 30B6+ cells were distributed throughout the gonadal primordia (Fig. 8 C-C').

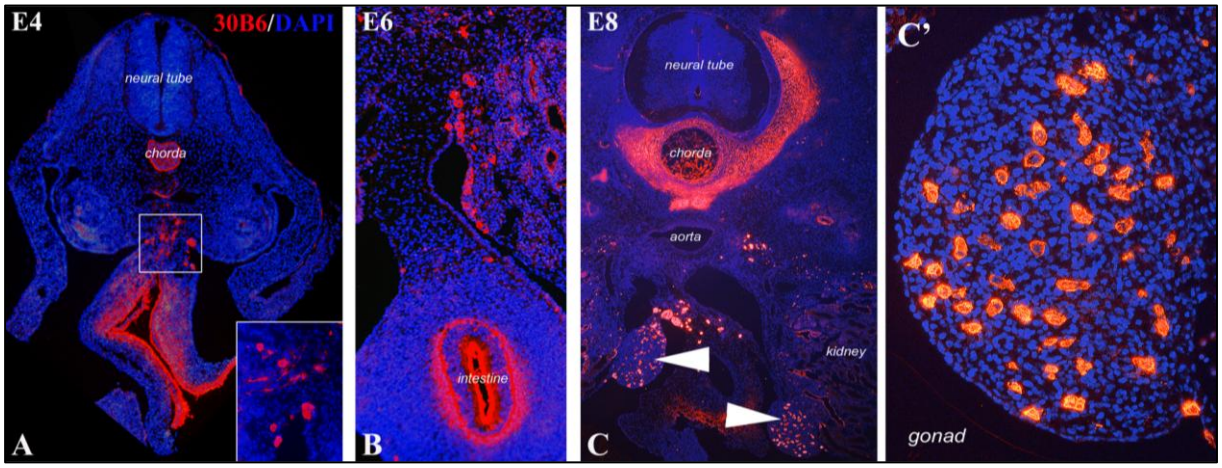

Figure 8. PGC migration to the developing gonads in 4-, 6-, and 8-day-old embryos. (A) Migration at E4; (B) colonization at E6; (C) gonadal population at E8.

3.1. 30B6-positive PGCs in the late embryonic gonads.

We further examined 30B6+ PGCs during late-stage embryonic gonad development. Sex-specific gonadal differentiation becomes apparent around HH26 (E5.5-E6) and morphologically distinct by E9 (Mizia et al., 2023). PGCs differentiate to either spermatogonia or oogonia, depending on gonadal microenvironment. At E10, a large number of 30B6+ cells showed uniform distribution within the gonads (Fig. 9 A), similar to DAZL+ cells (Fig. 9 A'). Double staining confirmed that most DAZL+ cells were also 30B6+, although DAZL+/30B6- cells were also present (Fig. 9 A”). Between E10 and E14, 30B6+ cell numbers decreased. At E14, 30B6+ PGCs clustered near the medial (presumptive cortical) surface (Fig. 9 B). By E18, 30B6+ cells were fewer (Fig. 9 C), consistent with testicular differentiation, as shown by well-defined medullary cords and a thinner cortex. These changes reflect key developmental events in sex determination, including reduced PGC numbers, spatial reorganization, and morphological alterations.

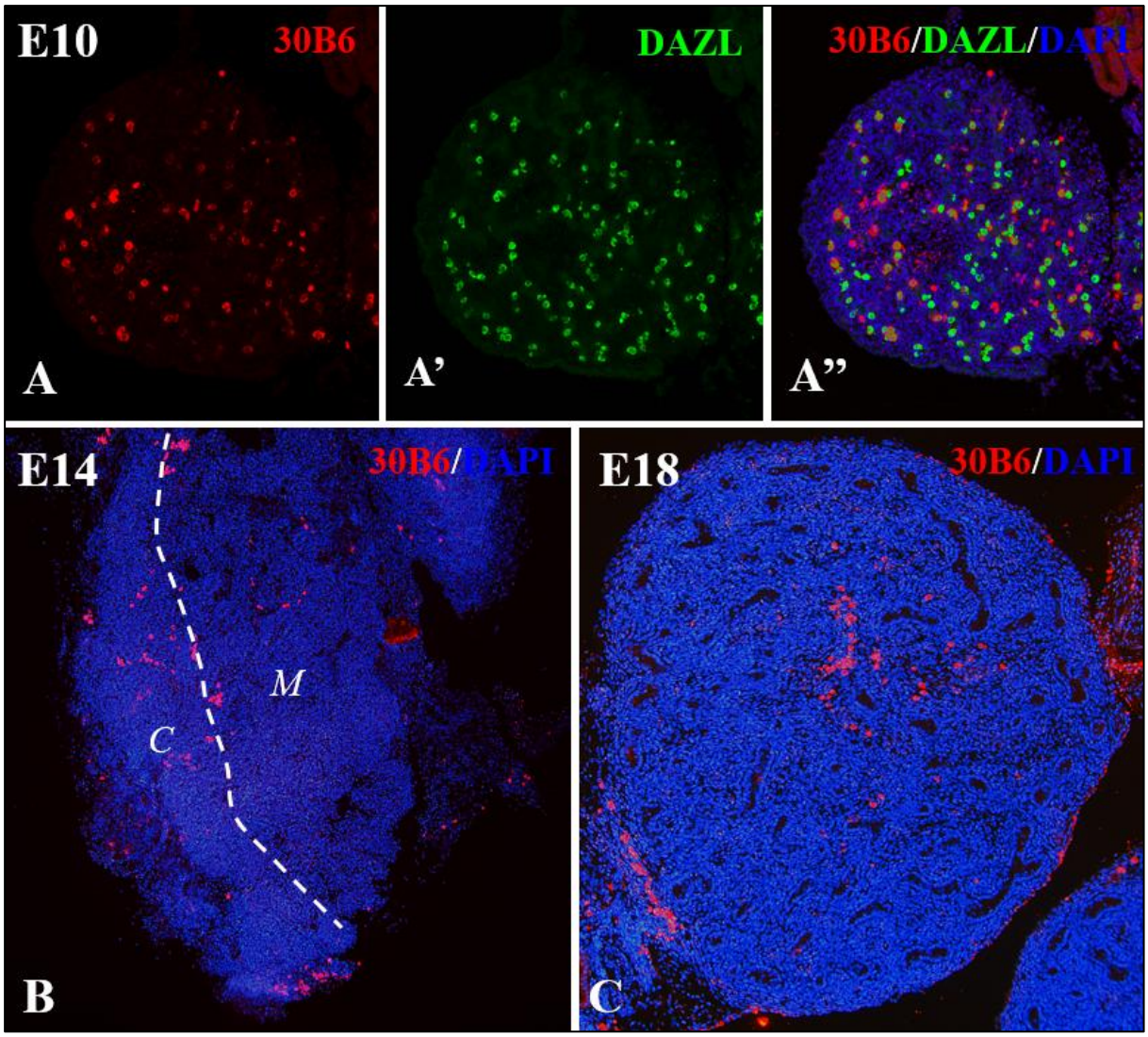

Figure 9. Immunostaining of embryonic gonads. At E10, 30B6+ cells co-express DAZL (A-A"). Later stages (E14-B, E18-C) show decreased signal intensity and region-specific localization consistent with sex differentiation.

4. Validation of 30B6 antibody specificity in avian PGC cultures and human embryonic tissues.

To confirm 30B6 specificity, we used chicken PGC cultures provided by Dr. Elen Gócza's laboratory. These cells were isolated from the circulation of 2.5–3-day-old embryos and cultured for 10 days (Fig. 10 A). Immunocytochemistry and confocal microscopy of cell cultures showed consistent membrane-associated 30B6 staining (Fig. 10 B, B", C, C"), contrasting with cytoplasmic DAZL (Fig. 10 B', B") and nuclear P63 (Fig. 10 C', C"). This distinct localization supports 30B6 as a reliable surface marker for avian PGCs.

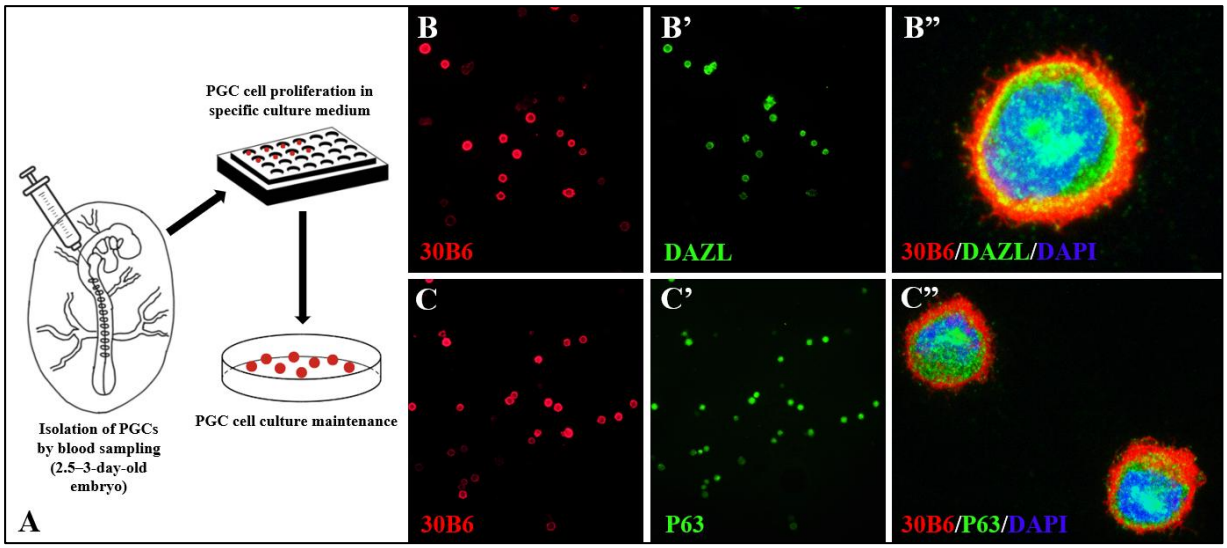

Figure 10. Double immunofluorescence of chicken PGC cultures. (A) Schematic illustration of PGC cell culture. (B-B") 30B6 colocalizes with DAZL (cytoplasmic) protein; (C-C") 30B6 also colocalizes with nuclear P63.

Finally, we tested the antibody on an 8-week human embryo. In gonad sections, 30B6+ PGCs exhibited cytoplasmic granular staining, differing from the membrane staining of chicken PGCs. Additionally, 30B6 immunoreactivity staining was observed in cartilage cells around the neural tube and differentiating pelvic skeleton (Fig. 11 A, B). Further studies are needed to clarify the developmental roles and molecular targets of 30B6.

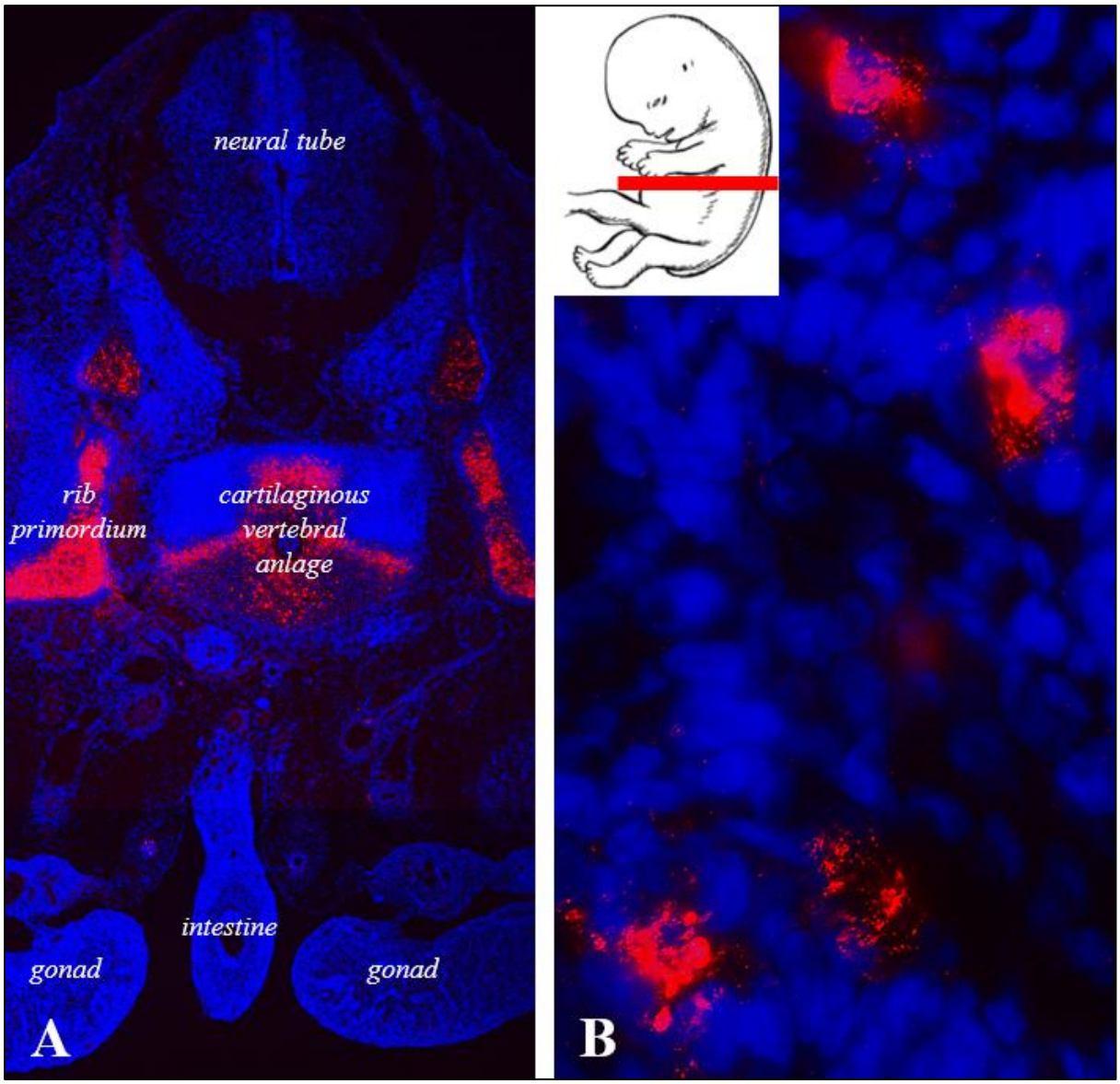

Figure 11. 30B6+ PGCs in an 8-week human embryo. (A) Cross-section of the gonads; (B) 30B6+ PGCs in the gonads.

Discussion

The development and characterization of novel monoclonal antibodies specific for primordial germ cells (PGCs) represent a critical advancement in developmental, reproductive, and conservation biology. This study provides the detailed characterization of the 30B6 mouse monoclonal antibody, revealing its dual specificity for avian B-cells and PGCs across various vertebrate species. The findings enhance our understanding of germ cell biology and offer practical applications in genetic preservation and developmental immunology research.

Preliminary investigations performed in our laboratory indicated that the 30B6 antibody exhibits specificity for guinea fowl antigens, recognizing both B-cells and PGCs. Accordingly, the initial step of my work focused on assessing its reactivity within the primary and secondary lymphoid organs of adult guinea fowl and chicken. In primary lymphoid organs, 30B6 antibody selectively labelled B-cells within the medullary regions of the bursa of Fabricius and the thymus. Regarding secondary lymphoid tissues, positive immunolabeling was observed within the peri-ellipsoidal white pulp and germinal centres of the spleen and caecal tonsil.

Analysis of embryonic chicken bursa of Fabricius tissues revealed distinct differences in staining patterns between 30B6 and B-cell-specific chB6 antibody, suggesting that 30B6 recognizes a cell surface molecule expressed during later stages of B-cell differentiation. Partial colocalization with CD44 and CD45 markers further supports the hypothesis that 30B6 can label mature and immature B-cells and a subset of migratory hematopoietic cells during early stages of lymphoid organ development. These findings demonstrate that the 30B6 antibody is a versatile tool for identifying a subpopulation of B-cells and migratory blood-borne progenitors.

Unexpectedly, 30B6 cross-reacted with mucin-producing goblet cells in non-avian species, including reptiles and humans. This suggests the antibody targets a conserved epitope shared by avian B-cell surface glycoproteins and vertebrate mucins, a finding that warrants biochemical characterization to resolve whether this reflects specific glycosylation patterns or structural homology. This cross-species reactivity broadens the potential application of the 30B6 antibody, from studying mucosal proteins to comparative experiments studying the role of mucin-like molecules in germ cell migration.

A key finding of my student research work is that 30B6 antibody is able to label PGCs in early chicken embryos (HH4) with membrane-specific staining, addressing a critical

limitation of existing markers like PAS staining, CXCR4, EMA-1, and SSEA-1 immunostaining, which lack cell-, and stage-specific reliability (Fig. 12). While DAZL and VASA-specific antibodies remain widely used markers, their cytoplasmic/nuclear localization limits live-cell applications. In contrast, membrane staining of the 30B6 enables potential use in fluorescence-activated cell sorting for isolating viable PGCs, a significant advantage for avian germline chimera production and *in vitro* culture systems. The partial colocalization of 30B6 antigen with DAZL in germinal crescent PGCs demonstrate the developmental heterogeneity within PGC populations. This supports the recent evidence about PGCs diversification during their migration and highlights the need for multiplex labelling to identify the developing PGCs.

The 30B6 antibody successfully labelled PGCs in chicken embryos at various developmental stages, producing a well-defined, membrane-associated signal on these cells. In contrast, the staining pattern in human embryonic and adult large intestine samples was predominantly cytoplasmic, indicating fundamental differences in the intracellular processing and localization of the target molecule between avian and mammalian species. The 30B6 antibody can label as early as the initial stages of embryonic development; however, further investigations are needed to confirm its expression before the HH4 stage (Fig. 12).

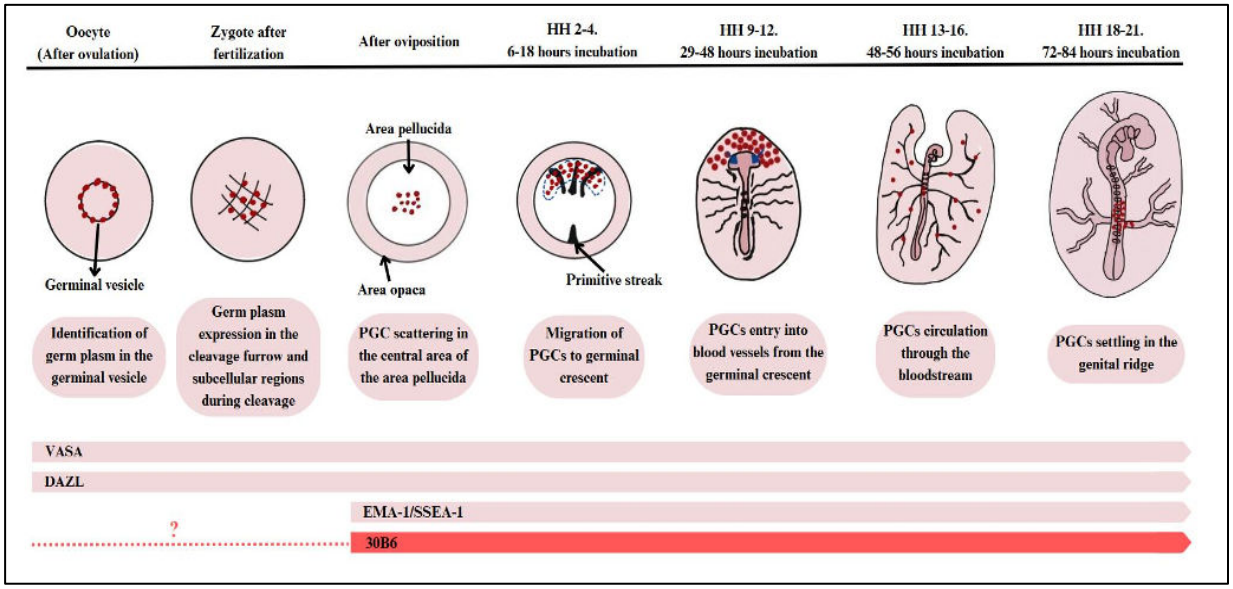

Figure 12. Migration of avian PGCs and labelling of different markers during embryonic development. The red dots represent PGCs migrating toward the developing gonadal ridges at various embryonic stages (the figure is the author's own work, modified after Kim and Han, 2018; Mathan et al., 2023).

The main limitation of my study is the unresolved molecular identity of the 30B6 antigen. In collaboration with Dr. Elen Gócza (Institute of Genetics and Biotechnology, Hungarian University of Agriculture and Life Sciences, Gödöllő) and Prof. Peter Balogh (Department of Immunology, University of Pécs), we performed biochemical analyses to determine the molecular weight of the 30B6 antigen. According to the preliminary results, the 30B6 monoclonal antibody recognizes a 250 kDa molecular weight protein. Functional studies (e.g. function blocking experiments) are needed to explore whether the 30B6 antigen plays active roles in B-cell or PGC migration/maturation.

In conclusion, the 30B6 monoclonal antibody is a versatile tool with dual applications in avian B-cell immunology and reproductive biology. Its ability to label B-cells and PGCs across species highlights its value in basic research and applied conservation. Future investigations should prioritize the precise identification and molecular characterization of the 30B6 target antigen, particularly emphasizing the biological implications of its membrane-bound and cytoplasmic forms in different species.

Summary

Primordial germ cells (PGCs) are the earliest precursors of gametes, appearing independently of germ layer formation during the very early stages of embryonic development. They play a crucial role in ensuring the transmission of genetic material to the next generation. Studying PGCs is fundamental to embryology, as it helps elucidate the early mechanisms of genital organ and tissue formation. Additionally, PGCs hold clinical relevance, offering potential future applications in treating infertility and genetic disorders. From an agricultural and conservation point of view, PGCs are critical for preserving genetic diversity in economically important species and for safeguarding endangered or indigenous animal populations.

In the present study, I characterized a newly developed mouse monoclonal antibody (clone name: 30B6) using immunocytochemical methods. This antibody (isotype: IgM) was originally developed in our laboratory to identify B-cells in the bursa of Fabricius. However, during our experiments, we unexpectedly observed that 30B6 also recognizes PGCs, both in the early embryo and developing gonads. The 30B6 antibody specifically marked B-cells within the developing bursa of Fabricius and identified a subpopulation of CD45+/CD44+ hematopoietic stem cells. Further analyses revealed that the 30B6 antibody cross-reacts with other species, including partridge, chameleon, and human intestinal tissues, particularly recognizing the goblet cells and their mucous product covering the intestinal epithelium. These findings suggest that 30B6 recognizes a structurally conserved epitope that may have different functional roles across various species and tissues. Regarding PGCs, 30B6 detected these cells as early as the HH4 stage of avian development and allowed tracking of their migration toward the gonads throughout embryogenesis. To confirm the PGC specificity of 30B6 antibody, double immunofluorescence staining was made using well-established markers such as DAZL, SSEA1, EMA1, and P63, further validating its efficiency in labelling chicken PGCs.

In conclusion, the newly developed 30B6 monoclonal antibody has proven to be a highly versatile tool capable of marking B-cells and identifying PGCs. This cell surface molecular marker provides a great opportunity for the precise isolation of PGCs and a better understanding of their developmental dynamics.

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Statement

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