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Unravelling early hematoendothelial development through the chick model: Insights and future perspectives

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A B S T R A C T

The chicken embryo has been an important model in advancing our understanding of early hematoendothelial development, particularly in the formation of hematopoietic stem cells (HSCs) and the endothelial-to-hematopoietic transition (EHT). The accessibility and ease of manipulation of chicken embryos have made them an invaluable tool for researching development of blood and endothelial cells. Early research using this model provided pivotal insights, demonstrating that intra-embryonic regions, such as the dorsal aorta (DA), are primary sources of HSCs, rather than the yolk sac (YS), as previously believed. The identification of intra-aortic hematopoietic clusters (IAHCs) and the process of EHT in the chicken embryo laid the foundation for similar discoveries in other vertebrate species, including mice and zebrafish. Recent advances in genetic tools, such as transgenic chickens expressing fluorescent proteins, have further enhanced the precision of cell lineage tracing and real-time imaging of dynamic cellular processes. This review highlights both historical contributions and contemporary advancements facilitated by the chicken model, underscoring its continued relevance in developmental biology. By examining key findings and methodological innovations, we aim to demonstrate the importance of the chicken embryo as a model system for understanding hematoendothelial development and its potential for informing therapeutic applications in regenerative medicine and blood disorders. Finally, we will underscore potential applications of the chicken model for comparative and omics-level studies in conjunction with other model systems and what future directions lie ahead.

1. Introduction

The development of blood and vascular systems, or hematoendothelial development, is a fundamental process in vertebrate embryogenesis, with far-reaching implications for understanding developmental biology and addressing clinical challenges, such as regenerative medicine and treatments for hematological disorders (Jaffredo et al., 2000; North et al., 2002; Tavian and Peault, 2005). One of the most significant models for studying early hematoendothelial development is the chicken embryo, which has been instrumental in biological research for over a century, particularly the pioneering work from Nicole Le Douarin and Francoise Dieterlen-Lievre whose studies used chimeras and investigated developmental hematopoiesis (Le Douarin, 1969; Dieterlen-Lievre, 1975; Martin et al., 1979). The ease of access to the chicken embryo *in ovo* while developing inside the egg or *ex vivo* in culture, and the flat configuration of the embryo on the yolk sac (YS) allows real-time observations and experimental manipulations through techniques such as electroporations (Harada et al., 2017), tissue grafting, bead implantation, cell lineage tracing and live imaging (Davey and Tickle, 2007; Mok et al., 2015; McColl et al., 2018). These unique attributes have been crucial in unravelling the early stages of blood and endothelial development (Le Douarin and Dieterlen-Lievre, 2013) especially the origins of hematopoietic stem cells (HSCs) from endothelial cells during embryogenesis (Jaffredo et al., 1998).

This review will examine the significant contributions of the chicken model to our understanding of early hematoendothelial development. We will explore the discovery of HSC origins, the role of the aorta in hematopoiesis, and the cellular and molecular mechanisms driving endothelial-to-hematopoietic transition (EHT). Additionally, this review will highlight how modern genetic tools and experimental methods are reinvigorating the use of the chicken model, underscoring its continued relevance in developmental biology and its potential for informing clinical applications, such as stem cell therapies and treatments for blood disorders.

2. Chicken as a model of hematoendothelial development

The chicken model is cost-effective, with eggs being inexpensive and easy to maintain, allowing for large-scale studies. Additionally, the relatively simple ethical considerations surrounding chick embryos make them a more accessible and flexible research tool compared to mammalian models. This aligns fully with the 3Rs framework model

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(Grimm et al., 2023) – replacement, reduction and refinement – to guide ethical research involving animals. In the broader context of vertebrate development, the chicken model complements studies in other organisms, offering unique insights into avian-specific processes and serving as a bridge between invertebrate models and mammals. In terms of the translational potential of using the chicken as a developmental biology model system, the sequencing of chicken genomes has also developed at rapid pace with new chicken genomes being annotated in addition to the original red jungle fowl reference genome (Rice et al., 2023; Huang et al., 2023). With the advancement of long-read sequencing technologies (mentioned later), the improvement to an annotated genome of different breeds will aid biologists focused on genomics as well as those interested in genetic diversity and breeding.

Avian models, such as the chick and quail, have been instrumental in shaping our understanding of developmental hematopoiesis over the past century, with many discoveries later applied to other vertebrate species (Jaffredo and Yvernogeau, 2014; Le Douarin and Dieterlen-Lievre, 2013; Nagai et al., 2018). The ease of accessibility of the avian embryo enabled critical studies, such as creating chick-quail chimeras to trace cell development (Le Douarin, 1969; Le Douarin et al., 2008). The grafting of quail embryos onto chicken yolk sacs supported the idea that HSCs primarily originate from intra-embryonic tissues rather than the yolk sac (Dieterlen-Lievre, 1975; Lassila et al., 1978, 1982; Cuadros et al., 1992), despite earlier theories suggesting the YS as the exclusive HSC source (Moore and Owen, 1967). Such YS chimera experiments provided the first conclusive evidence that cells found in the spleen and thymus 11 days post-grafting (such as granulocytes, erythrocytes, and lymphocytes) originated from intra-embryonic tissues, and not the YS (Dieterlen-Lievre, 1975). However, it is possible HSCs can also originate from YS-derived hemangioblasts that migrate into the presumptive dorsal aorta region before HH9 (Seco et al., 2020), which was the earliest grafting stage used in both chicken-to-quail and chicken-to-chicken chimera experiments. Furthermore, extra-embryonic cells are known to contribute to the dorsal aorta endothelium both in chick (Seco et al., 2020) and in mouse embryos (Tanaka et al., 2014), where they give rise to hemogenic endothelial cells and HSCs. The use of chicken-to-chicken YS chimeras has proved valuable by allowing B and T lymphocytes to be traced back to embryonic origins as they appeared 18 days post-grafting, whereas erythrocytes were observed 4 weeks after hatching (Lassila et al., 1978, 1982). Altogether these findings using chimera model systems have helped establish the importance of the YS during avian hematopoiesis. Indeed, later studies in chicken provided strong evidence that pinpointed the dorsal aorta as the primary source of HSCs and thus establishing the long-disputed intra-embryonic origin of the adult hematopoietic system (Cormier and Dieterlen-Lievre, 1988; Dieterlen-Lievre and Martin, 1981). More recently, the existence of bona fide HSCs in chicken originating from the dorsal aorta was demonstrated through an exquisite *in vivo* transplantation assay, by dissecting and tracing tissues from GFP donor embryos (aorta, yolk sac, allantois or head) and transplanting them into the chorio-allantoic membrane (CAM) of wild type recipient embryos (Yvernogeau and Robin, 2017).

3. Overview of chick primitive hematoendothelial development

The chicken embryo serves as an invaluable model for studying the early stages of hematopoietic and vascular development, particularly during primitive hematopoiesis (Sheng, 2010). The blood and vascular system in the chick embryo consists of blood cells, vascular endothelial cells, vascular smooth muscle cells, and the heart which originate predominantly from the lateral plate mesoderm (Prummel et al., 2020). The heart plays an essential role in the overall cardiovascular system and has been reviewed extensively elsewhere (Wittig and Munsterberg, 2016, 2020). Primitive hematopoiesis in the chick embryo represents the earliest wave of blood cell development, primarily occurring in the extra-embryonic YS (Sheng, 2010). This process is distinct from definitive hematopoiesis, which later generates long-term, self-renewing HSCs (Yvernogeau and Robin, 2017).

In chick embryos, hematopoietic cells derive from mesodermal cells that can be identified in the posterior primitive streak at HH4 (Hamburger and Hamilton, 1951) during the early onset of gastrulation (Seco et al., 2020) (Fig. 1). These progenitors, termed hemangioblasts or hematoendothelial progenitors, are bipotential cells that can differentiate into both blood and endothelial lineages (Nakazawa et al., 2006; Sheng, 2010). The existence of hemangioblasts was first proposed at the start of the 20th century (Sabin, 1920; Murray, 1932). The hemangioblasts hypothesis was supported by studies in chick embryos, where some gastrulating mesodermal cells were shown to express VEGF receptor 2 (VEGR2), a marker of early hemangioblasts (Xiong, 2008; Eichmann et al., 1993). These cells leave the epiblast through an epithelial-to-mesenchymal transition (EMT) (Voiculescu et al., 2007) and contribute to the splanchnic lateral plate mesoderm both in the extra-embryonic YS and the intra-embryonic region (Fig. 1).

Some cells that migrate into the extra-embryonic regions will start forming blood islands and have been speculated to be guided by extracellular matrix vacuoles and form a meshwork supported by N-cadherin-mediated cell-cell adhesion (Nakaya et al., 2022). Amongst the intra-embryonic splanchnic lateral plate mesoderm cells, there are progenitors that give rise to the dorsal aorta endothelium, including a subpopulation that will form the ventral hemogenic endothelium (Gritz and Hirschi, 2016) (Fig. 2). The dorsal endothelium of the dorsal aorta is initially derived from the splanchnopleura (Jaffredo et al., 2000) but becomes progressively replaced by paraxial mesoderm-derived progenitors. However, it has been shown that paraxial mesoderm-derived cells do not contribute to the ventral endothelium nor to HSCs (Pouget et al., 2006).

Primitive hematopoiesis begins with the formation of hemangioblasts/hematoendothelial progenitors (HEPs), which aggregate in the extra-embryonic YS to create blood islands. These blood islands are the primary sites where early blood vessel and blood cells originate from, including endothelial cells, primitive erythrocytes, megakaryocytes and macrophage precursors (Nakazawa et al., 2006). This process is tightly regulated by signalling pathways such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Wingless/Inegrated (Wnt), which influence the differentiation of mesodermal cells into either hematopoietic or endothelial lineages (Alev et al., 2010). Wnt signalling is required for VEGR2+ mesoderm cells to give rise to hematopoietic progenitors and BMP displays strong posteriorising effect on the same cells (Nostro et al., 2008). In zebrafish, FGF prevents presomitic mesoderm from adopting endothelial fate whilst BMP is required for endothelial specification (Row et al., 2018).

In chicken, hemangioblasts and HEPs express critical transcription factors that influence their cell fate decisions. Npas4l (previously known as Cloche) is a master regulator of hematoendothelial development, promoting the specification of both blood and endothelial lineages (Weng et al., 2007, 2020; Reischauer et al., 2016) and acting upstream of key hematoendothelial transcription factors Scl/Tal1 (T-cell acute lymphocytic leukemia 1) and Lmo2 (LIM domain only 2) (Marass et al., 2019). Scl/Tal1 is primarily associated with promoting hematopoietic fate by activating genes necessary for blood cell development while inhibiting endothelial differentiation (Patterson et al., 2007). Conversely, Lmo2 plays a dual role in maintaining hemangioblast identity and supporting both hematopoietic and endothelial differentiation, acting as a co-factor in transcriptional complexes that fine-tune lineage commitment (Patterson et al., 2007). Additionally, FGF signalling contributes to this fate determination by enhancing endothelial specification while suppressing hematopoietic potential, contributing to the early segregation of lineages in the chick embryo (Nakazawa et al., 2006).

Npas4l is not present in mammals, although the alternative gene which seems to have a similar role is Etv2, which itself is not conserved in avian species (Weng et al., 2020). In zebrafish, homozygous knockout

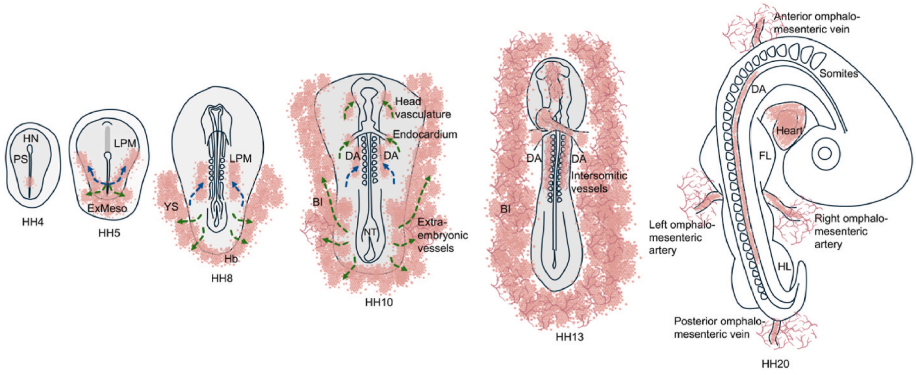

Fig. 1. Vascular development in the early chick embryo. This figure shows the progressive development of the vascular system in the chick embryo from early stages (HH4) through more advanced stages (HH20), highlighting the formation of major blood vessels, hemangioblasts (Hb) or HEPs, and extra-embryonic vasculature. At HH4, the primitive streak (PS) is visible, and Henson's node (HN) is forming and mesoderm begins to differentiate. At HH5, the lateral plate mesoderm (LPM) starts to expand as the mesoderm continues to organise, preparing for the emergence of early vasculature in the extra-embryonic mesoderm (ExMeso). At HH8, hemangioblasts, precursor cells to both endothelial and hematopoietic lineages, are migrating to the ExMeso forming within the yolk sac (YS) and lateral plate mesoderm. Early blood islands (BI) start to emerge and contribute to the circulatory system with primitive blood cells essential for transporting oxygen and nutrients to the embryo. At HH10, the dorsal aorta (DA) can be observed and endocardium are also forming in the heart, with visible head vasculature. Extra-embryonic vessels continue to develop outside the embryo whilst BIs become more prominent. At HH13, the LPM further develops, with the DA now fully formed and starting to fusion the posterior end and additional vascular structures visible, including intra- and extra-embryonic vessels. At HH20, the vascular system is fully developed, with distinct arteries and veins forming. The heart is well-defined, as are the somites, forelimb (FL), hindlimb (HL), and other major embryonic structures. The anterior and posterior omphalomesenteric arteries and veins are now visible, connecting the embryonic circulatory system with the extra-embryonic structures.

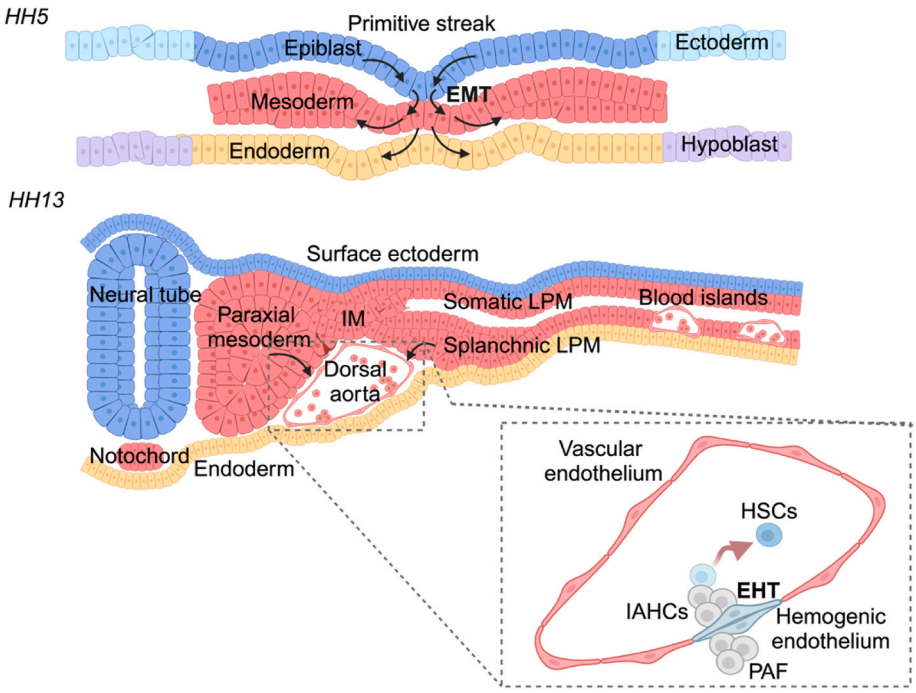

Fig. 2. Germ layer formation and differentiation during gastrulation and formation of the dorsal aorta. Top panel (HH5): during gastrulation, mesoderm cells (red) and endoderm cells (yellow) are formed through epithelial-to-mesenchymal transition (EMT) as cells from the epiblast (dark blue) ingress between the ectoderm (light blue) and hypoblast (purple). Bottom panel (HH13): Cross-sectional view of the embryo shows different mesoderm progenitors (red). Paraxial mesoderm, gives rise to musculoskeletal structures, dermis, intervertebral blood vessels, meninges and endothelial wall of the dorsal aorta (DA). The intermediate mesoderm (IM), situated between the paraxial mesoderm and the lateral plate mesoderm (LPM), contributes to the formation of the urogenital system. The LPM is divided into the somatic (dorsal) mesoderm and the splanchnic (ventral) mesoderm. The splanchnic LPM is involved in the formation of the vascular system which includes the blood islands, heart and DA. Somatic LPM develops parts of the body wall, such as the lining of the coelomic cavities, and of the limbs, such as the tendons and ligaments. Dotted box: a detailed view of the formation of hematopoietic stem cells (HSCs) via the intra-aortic hematopoietic clusters (IAHCs) from the hemogenic endothelium in the DA. Figure created in Biorender. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of Npas4l caused complete disruption of the vascular system (Reischauer et al., 2016) and further transcriptomic profiling of mutant embryos showed reduction in key markers for both endothelial and hematopoietic lineages (Marass et al., 2019). However, in the same study it was demonstrated that only endothelial lineages can be rescued by Npas4l overexpression thus suggesting Npas4l alone cannot induce hematopoietic lineages. These findings open up key questions about the evolution of the hematoendothelial system and how the initiation of such a key organ system is controlled by different genes between avians/fish and mammals. The divergence of Etv2 between mammals and other

vertebrates suggests either that it was once present in all vertebrate species and was lost in the avian system, or that the transcription factor was uniquely acquired by mammals to support hematoendothelial development. Over-expression of Etv2 in the chicken embryo drove the specification of the mesoderm towards an endothelial lineage, confirmed by the upregulation of Lmo2, but did not affect blood cell production (Weng et al., 2024). Increased dosage of Etv2 guides hemangioblasts/HEPs towards a hemogenic endothelial fate, whereas lower levels drive endothelial cell production, also confirmed using zebrafish studies (Zhao et al., 2022). The seemingly important evolutionary role of Etv2 therefore prompts deeper inquiry into the compensation mechanisms in species where this factor is not present, including chickens.

One of the key transcription factors that drive erythroid differentiation is Gata1. Disrupting Gata1 causes maturational arrest of both primitive and definitive erythroid lineages (Fujiwara et al., 1996; Pevny et al., 1995). Notably, distinct functional domains of Gata1 are needed to activate target genes in primitive and definitive erythroid cells, indicating that Gata1 may engage with different transcriptional partners in these lineages (Shimizu et al., 2001). Scl/Tal1, which is a helix-loop-helix transcription factor, and Lmo2 and Ldb1 (both LIM domain-containing transcription factors) form a multiprotein complex with Gata1 in definitive erythroid cells (Wadman et al., 1997). Their importance during embryo development is highlighted by complete absence of erythroid cells and embryonic lethality when these genes are disrupted (Shivdasani et al., 1995; Robb et al., 1995).

The existence of bipotent hemangioblasts has been controversial as there have been contradictive, and slightly flawed, studies disproving their existence in mouse embryo development (Ueno and Weissman, 2006; Padron-Barthe et al., 2014). Although these studies in mice made claims of the non-existence of the bona fide hemangioblast, both had technical and interpretational flaws that did not support their arguments, such as utilising reporter lines not specific to the early hemangioblast population. This topic has been extensively reviewed by others (Lacaud and Kouskoff, 2017) and most recently the existence of the elusive hemangioblast was proven in the mouse by utilising *in vivo* cellular barcoding to trace the lineages of mesodermal derivatives of the YS (Biben et al., 2023). Although demonstrating the existence of the hemangioblast in the mouse YS, the existence of these bi-potent cells within the embryo aorta has yet to been proven. This further demonstrates the complexity of deciphering the molecular mechanisms that exist from the onset of gastrulation to the formation of sub-populations of mesodermal cells defining their lineages. Initial evidence in the chick suggested that hemangioblasts, or HEPs, originate from the aortic endothelium, supported by the expression of Scl/Tal1 in endothelial lineages (Drake et al., 1997). In the mouse, further support comes from the observation that Runx1-positive Gata1-negative extra-embryonic mesoderm cells migrate to and contribute to the dorsal aorta endothelium (Tanaka et al., 2014).

By stage HH8 of chick embryo development, blood islands show signs of differentiation (Fig. 1), with some cells expressing red blood cell markers like hemoglobin genes, while others differentiate into endothelial cells (Nagai et al., 2018). This differentiation continues through stages HH8 and HH9, which is only approximately 5 h of development, where the blood and endothelial progenitors further proliferate and develop (Nakazawa et al., 2006). It has been described that larger blood islands, typically located at the periphery, tend to produce more blood cells, while medial blood islands closer to the embryo give rise to more endothelial cells (Nagai et al., 2018).

Cells in the blood islands give rise to erythro-myeloid progenitors (EMPs) which will go on to form the primitive erythrocytes, neutrophils, megakaryocytes and macrophages (Fig. 3) (Nagai et al., 2018; Tahara et al., 1983) as well as definitive erythrocytes at later stages (Nagai et al., 2018; Dardick and Setterfield, 1978). The endothelial cells from these

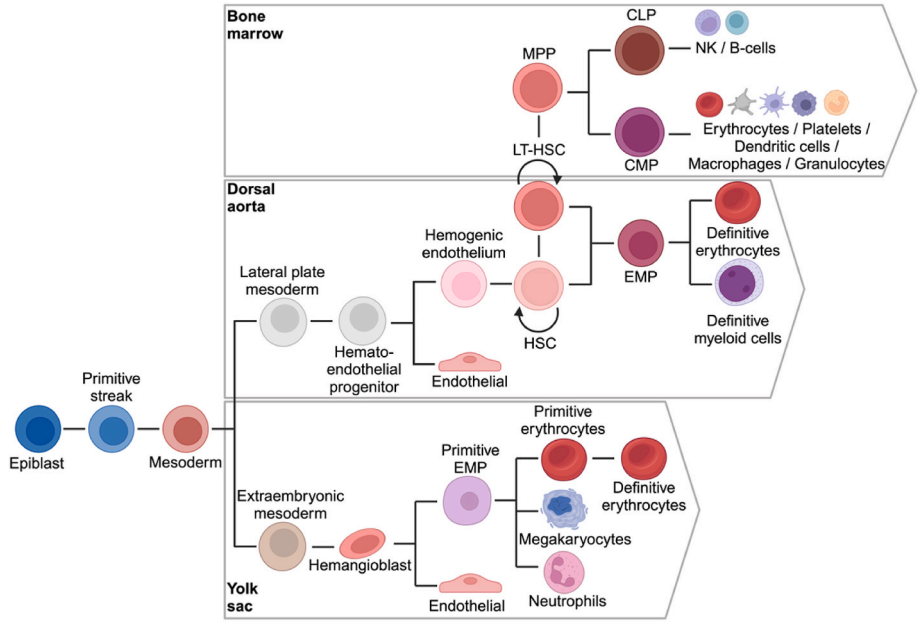

Fig. 3. Hematopoietic and endothelial lineage differentiation across different developmental niches. Formation of all cells in the vascular system originate from the epiblast, the primitive streak and then mesodermal cells during gastrulation. Yolk sac (YS, yellow box) is where primitive hematopoiesis occurs during the first wave. In the extra-embryonic mesoderm, hemangioblasts form to give rise to endothelial cells and primitive erythro-myeloid progenitors (EMPs) which further develop into the initial primitive erythrocytes and myeloid cells. The dorsal aorta (DA, light blue box) is where definitive hematopoiesis occurs within the embryo. Hemangioblasts/HEPs from the lateral plate mesoderm will form endothelial cells of intraembryonic blood vessels, such as the inter-somitic vessels and the DA, as well as the hemogenic endothelium in the ventral wall of the DA. Here, hematopoietic stem cells (HSCs) will give rise to EMPs and long-term HSCSs (LT-HSCs) which contribute to the fetal liver and bone marrow hematopoiesis. In the bone marrow (pink bow) LT-HSCs differentiate into multi-potent progenitors (MPPs), which further give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs differentiate into natural killer (NK) cells and B cells, while CMPs generate erythrocytes, platelets, dendritic cells, macrophages, and granulocytes. Figure created in Biorender. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

blood islands eventually integrate into the developing vascular system, forming a primitive vascular plexus, a network of early blood vessels which facilitates the transport of oxygen and nutrients via blood flow, and connects the YS to the embryo's heart (Nakaya et al., 2013). By stage HH10, molecular markers like Scl/Tal1, which is specific to the blood lineage, and Lmo2, which marks both blood and endothelial cells, help to further distinguish between these populations (Shin et al., 2009). As the embryo progresses to stages HH10-11, the heart begins to function and starts beating (Hogers et al., 1995), and a fully formed vascular system is established, allowing blood cells to circulate freely (Fig. 1). This system is critical for supplying oxygen and nutrients to the developing embryo, with the large, nucleated primitive erythrocytes generated through primitive hematopoiesis playing a vital role in early oxygenation (Shin et al., 2009). Cells that do not form part of the hematopoietic and endothelial system differentiate into mesothelial cells, marked by Hand2 expression, that typically form vascular smooth muscle cells (VSMCs), which line the developing blood vessels (Prummel et al., 2020).

4. Overview of HSC origin in the developing chicken embryo

Definitive hematopoiesis involves the generation of long-term, self-renewing HSCs, distinguishing it from primitive hematopoiesis, which primarily produces short-lived nucleated erythrocytes (Fig. 3) (Minko et al., 2003; Nakazawa et al., 2006), as well as neutrophils and macrophages (Balic et al., 2014; Cuadros et al., 1992). While primitive hematopoiesis supports the initial oxygenation for embryonic growth, definitive hematopoiesis ensures continuous production of the diverse spectrum of blood cells required throughout the life of the organism.

During embryonic development, definitive hematopoiesis occurs in two waves: the first wave is in the YS from erythro-myeloid progenitors (EMPs) - which gives rise to macrophages and microglia amongst other definitive hematopoietic cells – and the second wave from HSCs arising from the ventral wall of the dorsal aorta, endocardium and large blood vessels located in the head arteries and vitelline and umbilical arteries. Moreover, the main secondary hematopoietic organs in the developing chick include the spleen, thymus and Bursa of Fabricius (Jaffredo and Yvernogeau, 2014; Elsaid et al., 2020).

In mouse and human development, a great deal of attention has focussed on the formation of HSCs occurring in a region called aorta-gonad-mesonephros (AGM) (de Bruijn et al., 2000; Ivanovs et al., 2020). In chick, however, HSC formation occurs in the DA, rather than in the gonad or mesonephros at around stage HH12, and by stage HH17 the hemogenic endothelial cells line the ventral domain of the aorta throughout the embryo (Elsaid et al., 2020; Dieterlen-Lievre et al., 1988). At HH12 the vascular plexus, connected to the dorsal aortae (Figs. 1 and 2) as well as the endocardium and yolk sac vasculature, transforms into the vitelline arteries and veins following a process of vessel fusion, to allow full connection with the yolk sac blood islands (Le Noble et al., 2004). The formation and fusion of the dorsal aorta has been well documented (Pardanaud et al., 1987; Coffin and Poole, 1988) where the mechanisms are, overall, conserved with some differences between mammals, fish and birds (Yvernogeau et al., 2023). It is within the ventral wall of the dorsal aorta that cells undergo EHT to become multipotent HSCs (Jaffredo et al., 1998) and are considered to be the equivalent to HSCs found in zebrafish and mouse (Elsaid et al., 2020). At least in fish and chicken, this transition has been well researched and has been demonstrated to be tightly regulated by Notch, Wnt and TGFβ signalling that control the differentiation and maturation of HSCs (Bertrand et al., 2010; Clements and Traver, 2013; Lempereur et al., 2018; Monteiro et al., 2016; Wang et al., 2018). A conserved network of genes is expressed in hemogenic endothelial cells (HECs). For example, Runx1 is essential for definitive HSC development (North et al., 2002; Lacaud et al., 2002). Both Gata2 and Scl/Tal1 are expressed in HECs (Caprioli et al., 2001; Nagai et al., 2018), yet Scl/Tal1 has been demonstrated to be critical for HSC specification and survival (Porcher et al., 1996; Shivdasani et al., 1995) rather than directly driving EHT.

A critical observation in hematopoiesis was the identification of hematopoietic clusters, later termed intra-aortic hematopoietic clusters (IAHCs), which were first noted in chicken embryos in the early 20th century (Jordan, 1917; Dantschakoff, 1908) with subsequent studies more recently describing them in greater detail (Dieterlen-Lievre et al., 2006). In fact, it was Vera Danchakoff's pioneering work in the avian system that paved the way for understanding the true definition of stem cells, especially HSCs, which she termed 'hemoblasts'. The observation of these clusters, found closely attached to the aortic wall, are a common feature in the early development of nearly all vertebrate embryos such as human (Tavian et al., 1996), zebrafish (North et al., 2007), xenopus (Walmsley et al., 2002) and mouse (Garcia-Porrero et al., 1995).

The importance of the key hematopoietic transcription factor, Runx1, in definitive hematopoiesis is highlighted by studies in mice where Runx1−/− embryos lack HSCs but still contain primitive erythroid cells, and thus supports the idea that the IAHCs generate HSCs (Lacaud et al., 2002).

During development, IAHCs are directly derived from the HE which is part of the ventral wall of the DA (Fig. 2) (Jaffredo et al., 2000). This process has been observed in chick, fish and mouse where live imaging and lineage-tracing experiments show IAHC formation through EHT (Bertrand et al., 2010; Boisset et al., 2010; Seco et al., 2020). In mice, IAHCs peak at embryonic day 10.5 with approximately 700 cells per aorta and then decline by day 14.5 (Yokomizo and Dzierzak, 2010). Similarly, in chicken, a transient peak in IAHC cell numbers is observed at stage HH21 (Yvernogeau and Robin, 2017). These observations - namely, the transient formation and subsequent regression of IAHCs at critical developmental stages - are consistent across species. This temporal pattern supports the idea that the dorsal aorta is the site where definitive HSCs emerge, as the formation of IAHCs correlates with the window during which definitive HSCs are specified and released into circulation.

In avian models, it has even been possible to track IAHCs as they migrate beneath the DA, eventually forming structures known as para-aortic foci (PAFs) around day 6–7 of development (Jaffredo et al., 2000; Dieterlen-Lievre and Martin, 1981). These PAFs play a critical role in the early stages of hematopoiesis, as they give rise to progenitor cells that subsequently colonise major hematopoietic organs, including the thymus, spleen, bursa of Fabricius - a unique avian lymphoid organ located near the cloaca - and bone marrow (Lassila et al., 1979, 1980; Dunon et al., 1998, 1999). It has also been speculated that the PAFs are the avian equivalent to the fetal liver seen in other species (Saynajakangas et al., 2009). PAFs form the foundation for the development of the immune system and the structures have been extensively characterised in the chick model, highlighting its importance for establishing definitive hematopoiesis (Dieterlen-Lievre and Martin, 1981; Jaffredo et al., 2000; Dunon et al., 1998, 1999; Lassila et al., 1980).

In mammals, HSC functionality is typically assessed by transplanting cells into irradiated recipients (Cooper et al., 2023). In chickens, a similar transplantation approach is used, with donor cell engraftment and multilineage contribution monitored for up to 35 days post-transplantation (Lassila et al., 1979). However, this approach is uncommon in chicken and treatment with cyclophosphamide was used instead of irradiation. Furthermore, there is a requirement of a large number of chicken HSCs to perform the initial transplantation. A potential alternative approach for assessing hematopoietic functionality could involve transplanting embryonic tissues onto the CAM. This technique would enable the grafted tissue to connect with the host's vascular system - minimising donor cell colonisation - and supports tissue growth, thereby providing a controlled environment to evaluate the intrinsic hematopoietic activity of the transplanted cells. Furthermore, while the CAM assay is well established as a preclinical model in cancer and toxicology research (Kue et al., 2015), its application here offers a complementary strategy to traditional transplantation

experiments for studying hematopoietic stem cell potential. In developmental biology, transgenic chickens engineered to express fluorescent proteins have greatly simplified research, by enabling the effortless detection and tracking of donor cells. (Davey et al., 2018). The current development of transgenic lines, for both chicken and quails (Serralbo et al., 2020; Idoko-Akoh and McGrew, 2023), could aid answering key questions on where HSCs originate from, and from which region of the embryo, and propel the chick model closer to other model systems to allow long term lineage tracing of hematopoietic cells. It is worth noting that one of the main advantages of the chick embryo model system is using *in vivo* lineage tracing and live imaging to address developmental cell fate. Thus, the chicken embryo has been used to identify novel genes required for the formation and differentiation of hemangioblasts. Using a fluorescent reporter for an enhancer, flanking the Cer gene, labelling hemangioblasts (Teixeira et al., 2011; Zamir et al., 2017), cells were fluorescently sorted followed by transcriptomic microarray analysis (Serrado Marques et al., 2018). This study identified a previously unexplored gene, DIA1R, and uncovered its expression pattern throughout hematopoietic development. Although the transcriptomic dataset was captured using a bulk approach, it will still serve as a valuable resource to the community.

5. Future directions

Advancements in the technology surrounding genomics have allowed the chicken embryo to re-emerge as an attractive model organism in developmental biology. A fully annotated chicken genome is available however, improvements in genome annotation achieved by combining whole genome sequencing with long-read sequencing will aid methods relying on mapping reads to a comprehensive annotation, such as single-cell sequencing. Furthermore, these improvements will benefit functional studies such as knockdown experiments, where a well-annotated genome is vital for accurate targeting. The use of single-cell methods to identify and extrapolate genomic and transcriptomic information in a cell-type specific manner has proven useful particularly in developmental biology (reviewed in (Ton et al., 2020), where previously cells within an embryo of different lineages have proven difficult to distinguish and capture. Bulk sequencing often relies on capturing cell types based on limited marker information, and therefore does not consider the heterogeneity that lie within these populations. As a result of the emergence of single-cell RNA sequencing (scRNA-seq) technologies, notably from technologies such as 10X Genomics Chromium and Smart-Seq2 (Wang et al., 2021), several papers have already been published using the chicken, each targeting different biological systems within the developing embryo. Most of these have focussed on earlier stages of development (HH4-HH8) and have utilised dissection techniques to ensure that a significant proportion of their cell types captured are relevant to the system they are studying. For example, anterior dissections were used to investigate the emergence of different neural lineages across the neural plate border (Thiery et al., 2023; Williams et al., 2022), trunk dissections have been used to interrogate axial progenitors and notochord cells (Rito et al., 2023) and dissections of grafted somite progenitors were taken to decipher the contribution of these progenitors to the axial body (Busby et al., 2024). At later stages (HH25-HH31), scRNA-seq was performed to generate a comprehensive atlas of the developing limb (Feregrino et al., 2019). While a complete single-cell atlas of whole chicken embryos throughout development is not yet available—unlike the comprehensive datasets for zebrafish (Farnsworth et al., 2020), mouse (Pijuan-Sala et al., 2019) and human (Tyser et al., 2021) - the existing resources still offer valuable insights for deciphering key mechanisms in chicken embryogenesis, including gene expression dynamics, cell fate decisions, differentiation pathways, and gene regulatory networks (GRNs).

The intricate nature of regulatory networks in developmental biology has led to a focus not only on identifying which genes are active in various tissues during development but also on exploring how these genes are regulated. Ultimately, understanding epigenetic regulation in a cell-type specific manner can aid understanding of one of the most anticipated areas of embryo development; how cell fate decisions are made to determine lineage commitment in an embryo. The chicken embryo has proven to be a useful tool for looking at the epigenetics of blood biology. A leading reason for this is that erythrocytes in avian species remain nucleated, hence chromatin-level sequencing can be performed. A pivotal study in the field of epigenetics, using chicken red blood cells, demonstrated that genes were transcriptionally active in regions where chromatin was less condensed, in contrast to areas of chromatin where transcription was inactive (Weintraub and Groudine, 1976). Using deoxyribonuclease I (DNase I), they showed that active globin genes were preferentially digested in red blood cells in comparison to cells such as fibroblasts, or cells extracted from the brain. This established that chromatin structure is not merely a passive scaffold for DNA but plays an active role in regulating gene expression.

In the avian embryo, several efforts have been made across a multitude of cell types to identify novel *cis*-regulatory elements (CREs), typically enhancers, required for the regulation of genes in development (Mok et al., 2021; Patoori et al., 2020; Odaka et al., 2018; Tani-Matsuhana and Inoue, 2021). Regulatory elements in hematoendothelial development, notably those associated with master regulator genes, have also been identified, however these findings have mainly been restricted to non-avian organisms (Ogilvy et al., 2007; Grass et al., 2006; Delabesse et al., 2005). Part of the challenge has been identifying antibodies for histone marks to identify active regulatory elements (such as H3K27ac and H3K4me1 for enhancers) that worked in chicken, as conventional chromatin immunoprecipitation sequencing (ChIP-seq) required good antibodies and a high input of starting tissue material. Improvement to this technique and new antibodies has allowed ChIP-seq to be a useful tool in the avian community (Williams et al., 2024). However, as generating enough starting material remains problematic, the development of assay for transposase-accessible chromatin with sequencing (ATAC-seq) has looked to overcome this problem (Buenrostro et al., 2013). ATAC-seq is now a widely used technique to look at chromatin accessibility within different populations of cells. The maturation of single cell genomic techniques to include ATAC-seq has been a useful tool in developmental biology, to capture the chromatin landscape of individual cells in a heterogenous population. Although some technical challenges remain due to the amount of background noise present in ATAC-seq datasets of individual cells, there are multiple methods and downstream analyses available to counter these issues (reviewed in (Baek and Lee, 2020), and in chicken embryos single-cell ATAC-seq has already been used to investigate novel gene regulatory networks in neural crest populations (Williams et al., 2019). However, tissue-specific epigenetic regulation outside of these cell populations and across a broader range of stages remains unresolved, and further studies are required to overcome this, particularly for hematoendothelial development.

Alternative methods to examine the epigenetic landscapes, such as ChIP-seq, also include formaldehyde-assisted isolation of regulatory elements (FAIRE-seq) (Giresi et al., 2007). Both approaches were used in chicken to associate different chromatin characteristics with the expression of erythrocyte genes to try and understand the role of erythrocytes in the immune response across vertebrate species (Jahan et al., 2020). Although ATAC-seq has proven to be very useful, ChIP-seq offers a more focussed detail on protein-DNA interactions (Johnson et al., 2007), and FAIRE-seq can be used to highlight functionally active regulators which are free from nucleosomes (Giresi et al., 2007). To look more directly at regulatory elements and their interactions with promoter regions, 3D chromatin conformation, such as Hi-C, would be advantageous to use (Lieberman-Aiden et al., 2009). When considering the epigenetics of blood development, DNA methylation is known to have a crucial role in shaping cell fates. Whole Genome Bisulfite Sequencing (WGBS) is used to look at whole-genome patterns of DNA methylation (Cokus et al., 2008), and could be utilised to identify any

differentially methylated regions underpinning the transition of cells into hematopoietic cells, as well as how methylation of significant hematoendothelial genes, such as Tal1, Gata2 and Lmo2 mentioned previously, changes as blood and endothelial cells differentiate. The technique has previously been used to look at the formation of hematopoietic stem and progenitor populations in zebrafish (Li et al., 2022), and to identify how hypoxia influences the methylation landscape of blood cells in chickens (Zhang et al., 2018), however more work needs to be done to properly interrogate the mechanisms in which methylation influences cells to become hematoendothelial. A powerful tool for uncovering complex, poorly understood networks is the integration of multiple datasets using techniques looking at gene expression but also chromatin landscape. The ability to extract multiple modalities from single cells has been leveraged to look at previously uncovered mechanisms in lens development and disease (Tangeman et al., 2024). The method allows for more direct links between genes and open chromatin and can offer more accurate cell-type labelling given the two layers of information within each cell. Through pseudo-bulking analysis, novel regulatory elements, specific to distinct cell types can be found, which may have key roles in specialised cellular mechanisms. These same techniques could be used to extrapolate new elements, and therefore networks governing the different stages of embryonic hematopoiesis, such as the transition of mesodermal populations into the hemogenic endothelium, and eventually into HSCs. Such techniques have been used recently to investigate hematoendothelial development in human YS hematopoiesis (Goh et al., 2023), comparative EHT development between mouse and human (Mo et al., 2023) and endothelial origin of mouse hematopoiesis (Hou et al., 2024).

A common limitation of single-cell sequencing techniques is the loss of spatial information, as tissues are usually dissociated before library preparation. To combat this issue, cryosections throughout the chicken, along with mouse and zebrafish, embryo have been RNA sequenced and captured the transcriptomic information along the developing aorta (Yvernogeau et al., 2020). Integration of this type of spatial bulk information with single cell data has been used in separate chicken embryo studies to interrogate the tailbud and trunk (Mok et al., 2024) and the developing cardiac system (Mantri et al., 2021). New techniques that incorporate spatial information at single-cell resolution, like MERFISH (Chen et al., 2015) and 10X Genomics Xenium, are starting to be applied across various tissue types. However, no datasets are currently published using these techniques in the chicken embryo. These technologies will be critical for uncovering the mechanisms determining lineage decisions in heterogenous tissue such as that in hematoendothelial development, as well as extracting information about the microenvironment surrounding HSCs and their progenitors.

Another limitation of RNA sequencing is that short-read datasets typically provide only gene-level information. To achieve isoform-level resolution, longer-read sequencing technologies, such as those from Oxford Nanopore Technologies (Deamer et al., 2016) and PacBio (Wenger et al., 2019), are required. We predict that more studies will adopt long-read sequencing technologies to investigate the earliest stages of hematopoiesis, providing new insights into the commitment and differentiation processes of hemangioblasts and HEPs. As these methods start to advance and more data becomes publicly available, major findings are most likely to come not only from individual datasets, but also from the integration of several, whether that be just using chicken embryos, or across multiple species. Early adoption with this technique has already provided isoform-resolved transcriptome of the human preimplantation embryo (Torre et al., 2023). To truly understand early vertebrate hematoendothelial development, comparative studies across species will be essential. Through the integration of chicken datasets with those from other model organisms, it can be determined which hematoendothelial mechanisms are conserved and where instead there is variation across species. This comparison across different species will enable a broader understanding of hematopoiesis and will suggest where evolutionary adaptations have occurred. This sort of approach has already yielded novel insights in the conserved mechanisms in the formation of HSCs in the DA across different species using RNA-tomography (Yvernogeau et al., 2020).

It is noteworthy that the advancement of laboratory techniques to try and uncover regulatory elements, which could help with the discovery of novel GRNs in hematoendothelial development, is supported by the development of bioinformatic tools for single-cell methods such as SCENIC+ (Bravo Gonzalez-Blas et al., 2023). Such tools will be used to predict regulatory elements from multiomic datasets and help build complex GRNs. Deep-learning models and artificial intelligence (AI) has been introduced to assist with analysing the large genomic data that is becoming overwhelming (Alharbi and Rashid, 2022). These approaches will be instrumental with phylogenomics and comparative genomics to identify evolutionary conserved GRNs (Mo et al., 2024). Large datasets will require collaborative efforts hosted using cloud-based platforms, such as Terra, DNAnexus and Nextflow, to allow analysis to be performed on high-performance computers (Nagasaki et al., 2023; Koppad et al., 2021). Adoption of advanced cloud-based and big data technologies for processing and analysing omics data should provide new insights into hematoendothelial development and in hematological diseases.

Beyond only looking at the transcriptomics within hematoendothelial development, the protein expression landscape of the earliest cells committing to blood and endothelial lineages should be investigated, to not only see whether mRNA expression in these cells correlates with protein expression, but also to uncover the up-regulated proteins functioning to drive commitment decisions. Techniques interrogating the proteome have already proven invaluable when looking at avian embryo development, those such as mass spectroscopy, which has been used to look at the protein constituents in the CAM (Ahmed et al., 2022) and iTRAQ, a quantitative proteomic method previously used to study skeletal muscle formation (Ouyang et al., 2017). Combining proteomics with the multiomic techniques previously mentioned will provide clearer, more global insights into the mechanisms driving a hematoendothelial fate.

With the advancements of sequencing technologies, there has been a surge in developing gene editing technologies for the avian system. Notably, RNA-guided gene editing through the CRISPR/Cas9 system has proven to be powerful, highly precise method of investigating target genes *in vivo* by enabling genome modification across many different vertebrate species. These methods, combined with electroporation into early-stage embryos, has meant that the effect of gene deletion in somatic cells can easily be determined now in the chicken (Veron et al., 2015; Morin et al., 2017; Gandhi et al., 2017). Additionally, new methods focussing on *cis*-regulatory elements for gene regulation have enabled targeted epigenome editing (Williams et al., 2018) and enabled identification of functional roles of enhancers. These tools are now widely used and compliment the technologies of synthetic antisense oligonucleotides such as morpholinos or plasmid-based technologies.

Generating transgenic chickens has rather been more challenging than in mammals, fish and amphibians, as only a single oocyte can be retrieved from the mother hen. Initially, transgenic chicken production relies on the injection of viral DNA into the blastoderm (Idoko-Akoh and McGrew, 2023; Chapman et al., 2005), or the transfer of cells, such as modified primordial germ cells (PGCs) (Vick et al., 1993). Both methods have limitations, in that the effectiveness of the transgenic lines is still low (reviewed in (Bednarczyk et al., 2018)). However, the improvement to modifying PGCs and their reintroduction into hosts has allowed much greater potency in creating transgenics (Idoko-Akoh and McGrew, 2023). To date, only one transgenic chick line, Runx1-eGFP, has been created to trace hematopoietic cells (Davey et al., 2018) and one transgenic quail line, Tie1-eYFP, to evaluate endothelial cells (Sato et al., 2010). Although the Runx1-eGFP transgenic line of chickens exists, however it only labels monocytes and granulocytes, and cannot be used to visualise HSCs or their progenitors early in development. The creation of a transgenic reporter line where these cells could be labelled

would be invaluable in the study of the dynamic nature of hematopoiesis, to track how early in development cells are primed to become HSCs, and their migration patterns as well as long-term observations of the different waves of blood production. Lastly, there is a rapid growth of creating and using organoids for biomedical research (Yang et al., 2023) and this technology has been adopted in chicken to create intestinal organoids (Mitchell et al., 2024). The use of organoids will provide a further tool, especially in the avian model, to understand developmental processes particularly during different phases of hematopoiesis.

Although many techniques have been pioneered using other animal models, there are several research areas where the chicken model can play a leading role in uncovering key insights. For example, the chicken embryo offers a distinct advantage for studying erythrocyte enucleation. Unlike mammals, avian species retain nucleated erythrocytes during most of their lifespan, yet during specific developmental windows, enucleated erythrocytes are present (Yap and Zhang, 2021). This provides a unique opportunity to study the molecular and cellular mechanisms of enucleation *in vivo*, offering insights that could be translated to understanding erythropoiesis in mammals, including humans. (Bronnimann et al., 2018). The chicken also undergoes globin switching during development similar to humans (Qiu et al., 2008) thus making it an excellent model for studying the regulation of globin gene expression and the mechanisms that drive the switch from embryonic to adult hemoglobin. Altogether, the genomic approaches and future direction of using different technologies are summarised in Table 1.

In hematoendothelial development, one point of debate has been the existence of the hemangioblast, a bipotent progenitor which can differentiate into either blood or endothelial lineages. Recently, using the mouse embryo, hemangioblasts were observed to exist in YS blood formation (Biben et al., 2023). The next question would be to use the same lineage-tracing techniques to investigate the existence of the hemangioblast in the context of HSC formation in the DA. One potential method for lineage tracing in chickens, which has previously been applied to confirm the descendants of trunk neural crest cells (Tang et al., 2019) uses retroviruses to insert fluorescent markers into these cells, allowing the tracking of any cells which arise from the progenitors. Alternatively, a single-cell barcoding method could be used, as discussed by (Wagner and Klein, 2020) where unique DNA sequences are inserted into the genome of individual cells. As the cells divide, this approach allows the tracing of cell lineages back to their original ancestor. Although technically quite challenging, the incredible high precision of this approach makes it suitable for the confirmation, or contradiction, of hemangioblast existence.

6. Conclusion

The chicken remains a highly valuable model for advancing our understanding of early hematoendothelial development. It has been at the forefront in identifying key processes, such as the origin of HSCs from IAHCs and the mechanisms of EHT. Recent innovations, such as single cell multi-omics, the development of transgenic lines and advanced genome editing techniques and live imaging have greatly pushed our ability to study the dynamic processes in more detail during blood and vascular development. Additionally, enhancements in genome annotation will enable more in-depth analysis when combined with advancements in sequencing technologies, such as single-cell multi-omics, spatial transcriptomics, and long-read sequencing. This will now enable researchers to investigate gene expression and cellular interactions involved in hematoendothelial differentiation with greater resolution than ever before. In conclusion, the future of the avian embryo model system for investigating hematopoiesis, endothelial development, and uncovering novel insights looks bright.

CRediT authorship contribution statement

Lydia Pouncey: Writing – review & editing, Writing – original draft,

Table 1
Overview of modern genomic and functional techniques that can be used for developmental hematopoiesis research in the chick embryo.

Method	Purpose	Strengths	Limitations
Whole genome sequencing (DNA)	Identifying genetic variants, mutations, and structural changes	Provides a complete DNA sequence of a sample	High computational and storage demands, expensive
Chromatin accessibility (ATAC-seq)	Identifying open chromatin regions to infer regulatory elements	High resolution, low input requirements	Sensitive to cell lysis conditions, does not provide histone modifications
Chromatin profiling (ChIP-seq, Cut&Tag, Cut&Run)	Mapping protein-DNA interactions and histone modifications	High specificity for transcription factor binding	Requires high-quality antibodies, potential artefacts from fixation
Methylation (WGBS, EM-seq)	Studying DNA methylation at single-base resolution	High-resolution epigenetic analysis	High sequencing cost, complex data processing
Hi-C & 3D Chromatin Conformation Techniques	Maps genome architecture and enhancer-promoter interactions	Identifies topologically associated domains (TADs)	High computational demand, requires deep sequencing
Long-read Sequencing (Oxford Nanopore, PacBio HiFi)	Long-read sequencing to resolve structural variants	Improves genome assemblies, identifies complex variants	High error rate, requires high molecular weight DNA
Single-cell genomics (scRNA-seq + scATAC-seq, CITE-seq, ECCITE-seq)	Integrating transcriptomic and genomic profiles at a single-cell level	High resolution of cellular heterogeneity and simultaneous multi-layered molecular analysis	High cost, complex data analysis, potential technical noise
Transcriptome (bulk RNA-seq, RT-PCR)	Measuring gene expression at the RNA level	Captures dynamic gene expression changes	Does not provide spatial information, requires high-quality RNA
Spatial transcriptomics (MERFISH, 10X Xenium, RNA-tomography)	Mapping gene expression in spatial context within tissues	Preserves tissue architecture, enables spatial analysis	High computational demands, limited to known transcripts
Proteomics	Analysing protein expression and modifications	Provides functional insight beyond RNA levels	Requires advanced mass spectrometry, complex sample preparation
Deep Learning & AI in Genomics	Predicting regulatory elements, enhancer activity, and gene regulatory networks	Improves accuracy in large-scale data analysis	Requires computational resources and validated training data
Phylogenomics & Comparative Genomics	Identifies evolutionary conserved elements in avian species	Provides insight into adaptation and speciation	Requires high-quality genome assemblies
Cloud-Based Genomics Platforms (Terra, DNAnexus, Nextflow)	Scalable analysis of large genomic datasets	Enables collaborative and reproducible research	Requires internet access, may involve cloud storage costs
Genome editing (CRISPR/Cas9 and dCas9-	Targeted modulation of gene expression	Precise genetic perturbation,	Potential off-target effects,

(*continued on next page*)

Table 1 (*continued*)

Method	Purpose	Strengths	Limitations
Epigenetic Modifiers)	or via epigenetic marks	useful for functional studies	delivery challenges
Transgenic chicken lines	Generating genetically modified chickens to study gene function	Stable long-term gene expression studies	Time-consuming, requires extensive validation and breeding
Chimeric and Avian Organoid Models	*In vitro* modelling of avian tissue development	Enables tissue-specific studies and regenerative research	Limited organoid protocols for avian species
Lineage Tracing	Tracking the origin and fate of cells over time during development or disease	Reveals cell lineage relationships and developmental trajectories	Often requires genetic labelling or reporter constructs; limited resolution in complex tissues

This table summarises a range of methodologies used to analyse gene expression, chromatin dynamics, protein interactions, and computational approaches relevant to developmental biology research. Techniques such as single-cell genomics, whole genome sequencing, chromatin accessibility assays, chromatin profiling and spatial transcriptomics are highlighted alongside their primary purposes, strengths, and limitations. Molecular tools including CRISPR/Cas9 genome editing, lineage tracing and transgenic chicken models are also detailed, offering insights into targeted gene function studies. Computational strategies like deep learning in genomics and cloud-based platforms are included to address the increasing need for high-throughput data analysis. This comprehensive summary provides a framework for selecting appropriate techniques to investigate gene regulatory networks and functional genomics in chick hematoendothelial development.

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Competing interests

The authors declare no competing or financial interests.

Data availability

No data was used for the research described in the article.

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