Investigating the Role of Podocalyxin in Hematoendothelial Progenitor Development in the Chick Embryo

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Abstract

The extracellular matrix (ECM) plays a critical role in regulating cell fate decisions during early embryonic development. Hematoendothelial progenitors (HEPs) arise from the lateral plate mesoderm and give rise to both endothelial and hematopoietic lineages. Podocalyxin (PODXL) is an ECM-associated transmembrane protein implicated in cell adhesion, polarity, and migration; however, its role during early hematoendothelial development remains poorly understood.

In this study, we focus on characterizing the spatial and temporal expression of *Podxl*, during chick embryogenesis and to investigate its functional role during early hematoendothelial development. We also focused on its relationship with Tal1, a well- known hematopoietic transcription factor.

Through hybridization chain reaction (HCR) experiments, the spatial expression of *Podxl* and *Tal1* was analysed across key Hamburger- Hamilton (HH) stages. emerging from the LPM, where HEP cells reside. Splice-blocking morpholino's were used to knock down *PODXL* to perform functional analyses. PODXL expression was detected in the lateral plate mesoderm and developing cardiac regions during early embryogenesis, partially overlapping with Tal1 expression. Following *Podxl* knockdown, 46% of embryos exhibited heart looping defects and 15.3% displayed cardia bifida, indicating a requirement for *Podxl* in normal cardiac morphogenesis.

To elucidate regulatory mechanisms, enhancers of *PODXL* were cloned and injected into chick embryos. Selected based on single-cell data, several enhancers displayed strong tissue-specific activity in the lateral plate mesoderm, dorsal aorta, endothelium, and cardiac regions, indicating their role in driving *PODXL* expression during hematoendothelial and cardiovascular development.

These findings suggest that PODXL may influence hematoendothelial development indirectly by modulating the cellular microenvironment rather than acting as a direct regulator of Tal1 expression.

Overall, this study identifies Podocalyxin as an important ECM-associated factor during early chick development and provides a foundation for future studies investigating its mechanistic role in hematoendothelial lineage specification and cardiovascular development.

1.1 The chick as a model organism

The chick embryo (*Gallus gallus domesticus*) is a well-established model system for investigating early vertebrate development and offers numerous experimental advantages for studying developmental processes in amniote species. Its accessibility, robustness, and suitability for experimental manipulation have made it a central model in developmental biology.

The domestic chicken occupies a distinct evolutionary position that bridges lower vertebrates and mammals, making it particularly valuable for comparative and translational developmental studies. As a result, the chick embryo has been extensively used in classical experimental embryology (Rashidi and Sottile, 2009), as well as in studies investigating the molecular basis of cell development and cell–cell interactions (Weeke-Klimp *et al.*, 2010). In addition, the chick model has contributed significantly to research in experimental medicine (Ribatti, 2012), immunology, behaviour, reproduction, and epigenetics (Li *et al.*, 2011).

Several biological and practical characteristics make the chick embryo a desirable model organism. Phylogenetically, it is closer to mammals than alternative models such as zebrafish or nematode worms, and it possesses well-developed and well-characterised central nervous, cardiovascular, and respiratory systems. Chick embryos are conveniently sized, undergo a relatively short incubation period of approximately 21 days, and are easily accessible throughout development during both *in ovo* and *ex ovo* culture.

Chick development has been comprehensively described by Hamburger and Hamilton (1951), who established a staging system based on morphological criteria rather than incubation time. The Hamburger–Hamilton (HH) staging system remains the standard framework for describing chick embryogenesis and allows accurate comparison between embryos independent of incubation conditions. During the early HH stages, major organs and systems are established, followed by subsequent growth and maturation during later stages of development.

Early cardiovascular development in the chick embryo is rapid and well defined. Extraembryonic blood vessels first form in the yolk sac during early stages of development, followed shortly by the initiation of cardiac activity and the establishment of a functional circulatory system. These features make the chick embryo particularly well suited for studying early vascular and haematopoietic development.

A major experimental advantage of the chick model is the external development of the embryo, which allows direct access throughout embryogenesis and facilitates experimental manipulation (Mok *et al.*, 2015). The chick and human genomes share considerable homology, and early chick embryos exhibit morphological similarities to human embryos. Although the avian genome is smaller than the human genome and only approximately 60% of chicken genes have a traceable single human ortholog, avians possess approximately 20,000–23,000 protein-coding genes, a number comparable to that of mammals.

The flat morphology and mechanical robustness of the chick embryo facilitate grafting and transplantation experiments and allow long-term live imaging of developmental processes (Nájera and Weijer, 2020). At the time of egg laying, the embryo consists of a flat, two-layered blastoderm positioned on the surface of the yolk, making it highly accessible for microsurgical manipulation. This accessibility enables precise temporal control of experiments and supports efficient data collection.

Additional advantages include the ability to terminate incubation at defined developmental stages, allowing accurate staging and sample availability, as well as the embryo's suitability for micromanipulation even at early stages of development. Collectively, these features provide high experimental flexibility, short developmental timelines, and precise control over embryonic staging.

Despite these advantages, the chick embryo model also presents certain limitations. These include less standardisation in imaging and evaluation protocols compared to some mammalian systems and the fact that the embryo develops within a closed system, limiting excretion of administered substances. However, studies have demonstrated that classical pharmacokinetic profiles can still be observed for environmental pollutants and pharmaceutical compounds in chick embryos, highlighting the utility of this model for pharmacokinetic research despite these constraints.

Overall, the chick embryo represents a powerful and appropriate model for studying early embryonic development, particularly processes involving mesoderm patterning, cardiovascular formation, and the emergence of haematoendothelial progenitor populations.

Figure 1 summarises the main benefits of using the chick embryo model (Fig 1).

Figure 1- Benefits of using the avian chick embryo for studying developmental biology questions (Vilches-Moure 2019).

1.2 Overview of early chick development

The chick embryo is a particularly advantageous model for studying early amniote development due to its flat morphology, relative transparency, and external development. At the time of egg laying, the embryo consists of approximately 20,000–30,000 cells and lies on the surface of the yolk beneath the vitelline membrane (Serrano Nájera and Weijer, 2020). Figure 2 shows the overall organisation and orientation of the early chick embryo prior to gastrulation.

A subset of these cells forms the epiblast, a single-cell-layered, quasi-epithelial disc that gives rise to all three embryonic germ layers. At the periphery of the embryo, the epiblast overlies a rigid, multi-layered population of large mesenchymal cells that are in direct contact with the yolk (Vasiev *et al.*, 2010). This structural arrangement contributes to the mechanical properties of the blastoderm and influences early morphogenetic movements.

Morphologically, the early chick embryo exhibits a circular, flattened geometry and is organised into two concentric regions: the area opaca (AO) and the area pellucida (AP). The area opaca forms the outer region of the blastoderm and contributes primarily to extra-embryonic tissues, whereas the centrally located area pellucida contains the epiblast cells that will form the embryo proper. During early development, clusters of small, rounded cells attach to the ventral surface of the epiblast within the area pellucida to form the primary hypoblast. These cells subsequently spread and flatten to generate a continuous epithelial layer known as the hypoblast (Serrano Nájera and Weijer, 2020).

Although the hypoblast does not contribute directly to embryonic tissues, it plays a crucial signalling role during early patterning events. Both the hypoblast and the area opaca contribute to extra-embryonic structures, while the epiblast of the area pellucida generates all embryonic lineages. At the posterior lateral boundary between the area opaca and area pellucida lies a specialised band of epithelial cells known as the marginal zone. This region integrates signalling inputs that establish the anterior–posterior axis and define the site at which gastrulation will initiate (Serrano Nájera and Weijer, 2020).

Hypoblast cells are denser in the posterior region of the embryo, where they begin to fuse into an epithelial monolayer in a posterior-to-anterior manner beneath the epiblast, without extending into the marginal zone (Fig. 2). Posterior hypoblast cells later fuse with, and are displaced by, the endoblast, a population of cells arising from deeper layers beneath the posterior marginal zone (Vasiev *et al.*, 2010).

Mechanical forces also play an important role during early chick development. Only cells at the outer periphery of the area opaca attach to the vitelline membrane, generating tension as these cells extend protrusions and migrate radially outwards (Bellairs, Boyde and Heaysman, 1969). At the time of egg laying, the embryo contains approximately 50,000 cells and can be subdivided into at least four distinct populations: the area opaca, epiblast, marginal zone, and hypoblast, each characterised by specific lineage markers (Sheng, 2013; Serrano Nájera and Weijer, 2020).

These coordinated cellular, molecular, and mechanical processes establish the spatial framework required for gastrulation, the critical event that reorganises the epiblast into the three primary germ layers.

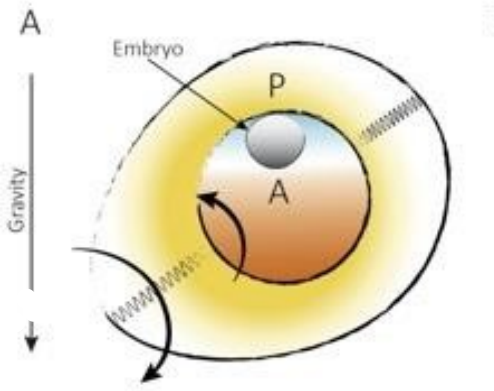

Figure 2- Figure 2. Early organisation of the chick embryo prior to gastrulation. (A) Orientation of the embryo on the yolk during intrauterine development. Figure adapted from Serrano Nájera and Weijer (2020).

1.3 Gastrulation

Gastrulation is a fundamental morphogenetic process during which the simple, single-layered epiblast is reorganised into a complex, multilayered embryo consisting of three primary germ layers: ectoderm, mesoderm, and endoderm (Serrano Nájera and Weijer, 2020). This process establishes the basic body plan of the embryo and provides the foundation for all subsequent tissue and organ development, representing a key evolutionary innovation in multicellular animals (Muhr and Ackerman, 2021).

In the chick embryo, gastrulation begins with the formation of the primitive streak, a transient structure that arises at the posterior region of the area pellucida. Primitive streak formation is initiated by coordinated cell movements within the epiblast, driven by large-scale vortical flows that direct mesendodermal precursor cells from the posterior marginal zone toward the embryonic midline (Vasiev *et al.*, 2010). These movements result in the thickening of the epiblast and the emergence of the primitive streak, which becomes visible at early Hamburger–Hamilton (HH) stages.

As the primitive streak forms and elongates, epiblast cells undergo epithelial-to-mesenchymal transition (EMT), a process in which cells lose apical–basal polarity and cell–cell adhesion, enabling them to ingress and migrate into deeper layers of the embryo (Chuai *et al.*, 2006) (Fig. 3). EMT is essential for germ layer formation and is tightly coordinated with continued recruitment of neighbouring epiblast cells into the streak.

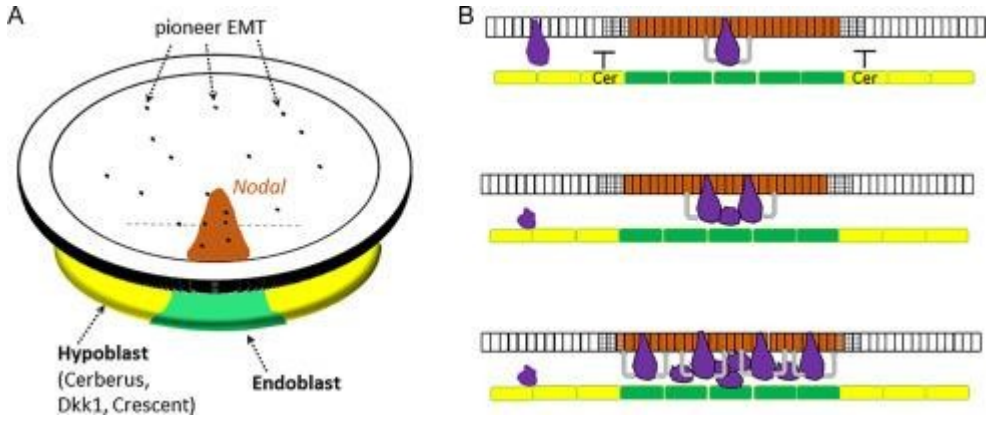

Figure 3- Initiation and maintenance of the primitive streak through epithelial-to-mesenchymal transition (EMT).
(A) Schematic showing pioneer epiblast cells undergoing EMT and ingressing at the posterior epiblast, coinciding with localised Nodal expression. Endoblast cells (green) displace the hypoblast, relieving repression of Nodal signalling.
(B) Sequential EMT events within the epiblast that drive cell ingression and progressive elongation of the primitive streak.(Adapted from Perea-Gomez and Meilhac, 2015.)

The elongation of the primitive streak occurs progressively in an anterior direction until it reaches its maximum length by HH4, at which point it spans approximately 80% of the diameter of the area pellucida (Rozbicki *et al.*, 2015; Saadaoui *et al.*, 2020). During this period, distinct regions along the primitive streak become molecularly patterned, allowing ingressing cells to be allocated to specific germ layer fates.

Gastrulation is regulated by the integration of multiple signalling pathways, including Wnt, Nodal, BMP, and FGF signalling. In the posterior region of the embryo, Wnt8c and Vg1 signalling promote primitive streak initiation, while graded BMP and Wnt inhibitor activity along the anterior–posterior axis refines streak patterning (Skromne and Stern, 2001; Wei and Mikawa, 2000). At the anterior tip of the streak, Hensen's node acquires organiser properties through the expression of transcription factors such as *goosecoid* and *chordin*, which antagonise BMP signalling and contribute to axis formation.

Morphologically, the primitive streak appears abruptly as a triangular structure at the posterior area pellucida, corresponding to HH stage 2. It then narrows and elongates, becoming parallel-sided by HH stage 3. Cells within the streak are highly dynamic, exhibiting coordinated migration, proliferation, and ingression as they move from the epiblast into deeper mesodermal and endodermal layers (Perea-Gomez and Meilhac, 2015). Mechanical tension generated by cell ingression drives large-scale tissue rearrangements across the epiblast, contributing to midline convergence and embryonic symmetry (Nájera and Weijer, 2023) (Fig. 4).

Cells that ingress through the primitive streak give rise to the mesoderm and definitive endoderm. As gastrulation concludes, the primitive streak regresses posteriorly, and the three germ layers become spatially organised. This marks the end of gastrulation and the transition to subsequent developmental processes, including neurulation, mesodermal patterning, and body axis elongation. Although gastrulation differs morphologically between vertebrate model systems, the underlying outcomes—germ layer formation and axis establishment—are conserved (Fig. 5).

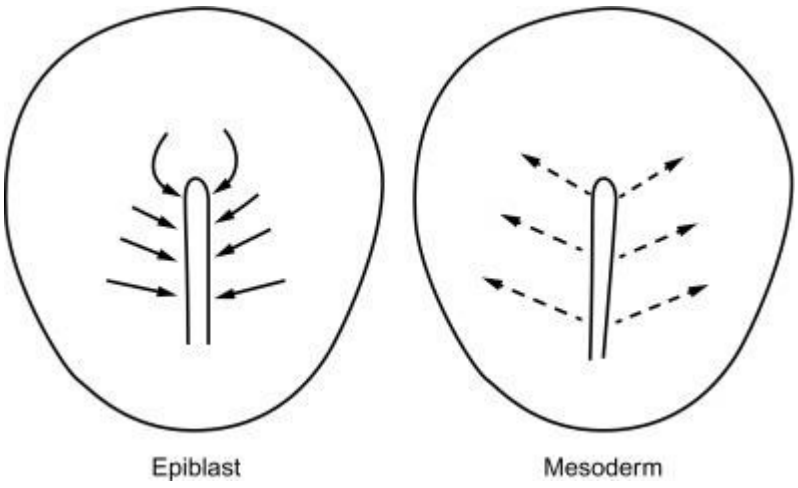

Figure 4. Tissue-scale cell movements during the full primitive streak stage.
Left: Convergent cell movements within the epiblast towards the primitive streak.
Right: Migration of mesodermal cells away from the primitive streak following ingression, contributing to germ layer organisation. (Adapted from Morizane and Bonventre, 2018.)

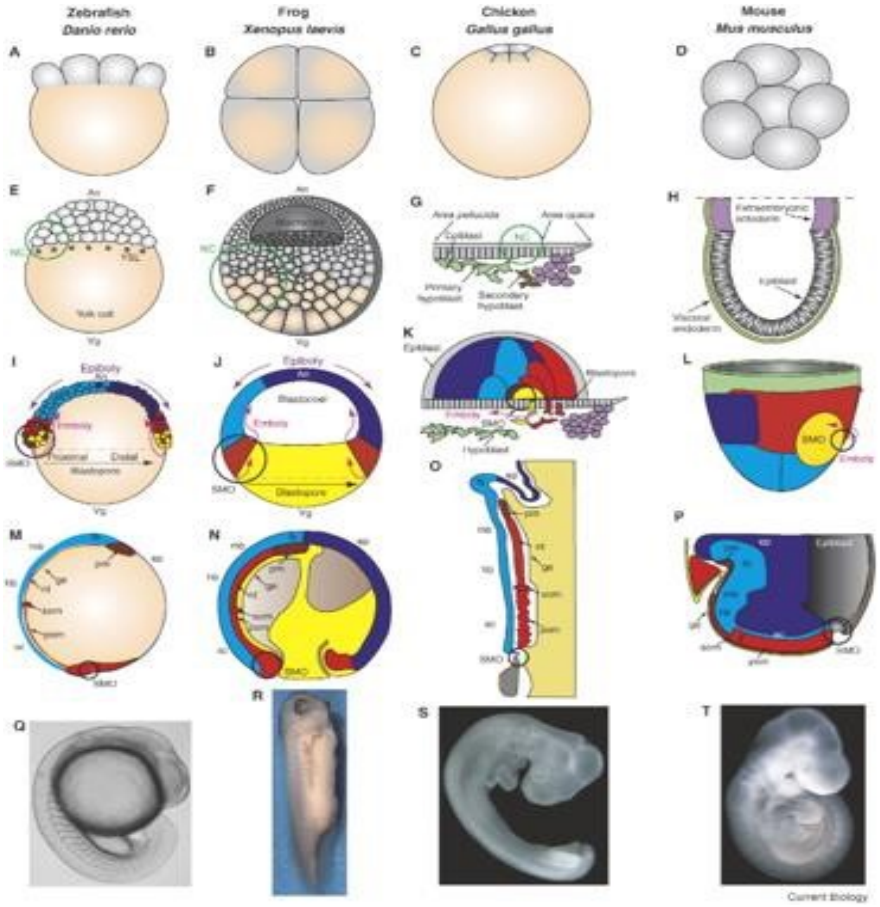

Figure 5. Comparative overview of gastrulation across vertebrate model organisms.
Schematic comparison of early embryonic development and gastrulation stages in zebrafish, Xenopus laevis, chick, and mouse, highlighting conserved outcomes such as germ layer formation and axis establishment despite species-specific morphological differences.
(Figure adapted from Solnica-Krezel, 2005b.)

1.4 The lateral plate mesoderm

Gastrulation establishes the three primary germ layers—ectoderm, mesoderm, and endoderm—which collectively form the foundation for all tissues and organs in the developing embryo. Following gastrulation, the mesoderm undergoes further spatial organisation along the mediolateral and anterior–posterior axes to generate distinct mesodermal subtypes, including the axial, paraxial, intermediate, and lateral plate mesoderm (Solnica-Krezel, 2005).

In chick embryos, the lateral plate mesoderm (LPM) emerges from mesodermal cells that ingress through posterior and mid-regions of the primitive streak and migrate laterally during and shortly after gastrulation. The LPM is defined by its position lateral to the somite-forming paraxial mesoderm and is composed of highly motile cells that undergo extensive morphogenetic rearrangements. Proper specification of the LPM is essential for the establishment of the body wall, limb buds, and the cardiovascular system, making it a critical contributor to overall body plan organisation and organogenesis.

Organisation of Mesodermal Territories

As mesodermal cells migrate away from the primitive streak, they are exposed to graded signalling environments that determine their regional identity. High levels of bone morphogenetic protein (BMP) signalling promote lateral and ventral mesodermal fates, whereas lower BMP activity favours paraxial mesoderm specification (Solnica-Krezel, 2005). In vertebrate embryos, including chick, BMP signalling acts in concert with Nodal to coordinate anterior–posterior and dorso-ventral patterning during early LPM formation (Prummel, Nieuwenhuize and Mosimann, 2020).

Experimental evidence from zebrafish and *Xenopus* demonstrates that increasing BMP activity expands LPM domains and enhances expression of genes associated with erythroid, vascular, and pronephric lineages (Hammerschmidt *et al.*, 1996; Prummel *et al.*, 2020). Additional signalling pathways, including fibroblast growth factor (FGF), canonical Wnt, and retinoic acid (RA), further refine LPM identity and regionalization, influencing the specification of cardiac, renal, and haematoendothelial progenitors (Prummel *et al.*, 2020).

Once specified, the LPM condenses into bilateral sheets of cells along the lateral edges of the embryo, classically referred to as the lateral plates (Fig. 6). These plates subsequently undergo further subdivision along both the anterior–posterior and mediolateral axes to generate distinct progenitor domains (Prummel *et al.*, 2020).

Morphogenesis and Subdivision of the LPM

During early somitogenesis, convergent extension movements contribute to the elongation and positioning of the LPM. These morphogenetic processes are regulated in part by planar cell polarity (PCP) signalling, which coordinates cell orientation and movement, ensuring correct alignment of the LPM relative to the paraxial mesoderm, ectoderm, and endoderm.

As development proceeds, the LPM splits into two distinct layers: the somatic (parietal) mesoderm adjacent to the ectoderm, and the splanchnic (visceral) mesoderm adjacent to the

endoderm (Fig. 6). The somatic mesoderm contributes to the formation of the body wall and limb connective tissues, whereas the splanchnic mesoderm gives rise to components of the cardiovascular system and internal organs (Prummel *et al.*, 2020). This subdivision is a key developmental milestone that underpins the functional diversity of LPM-derived tissues.

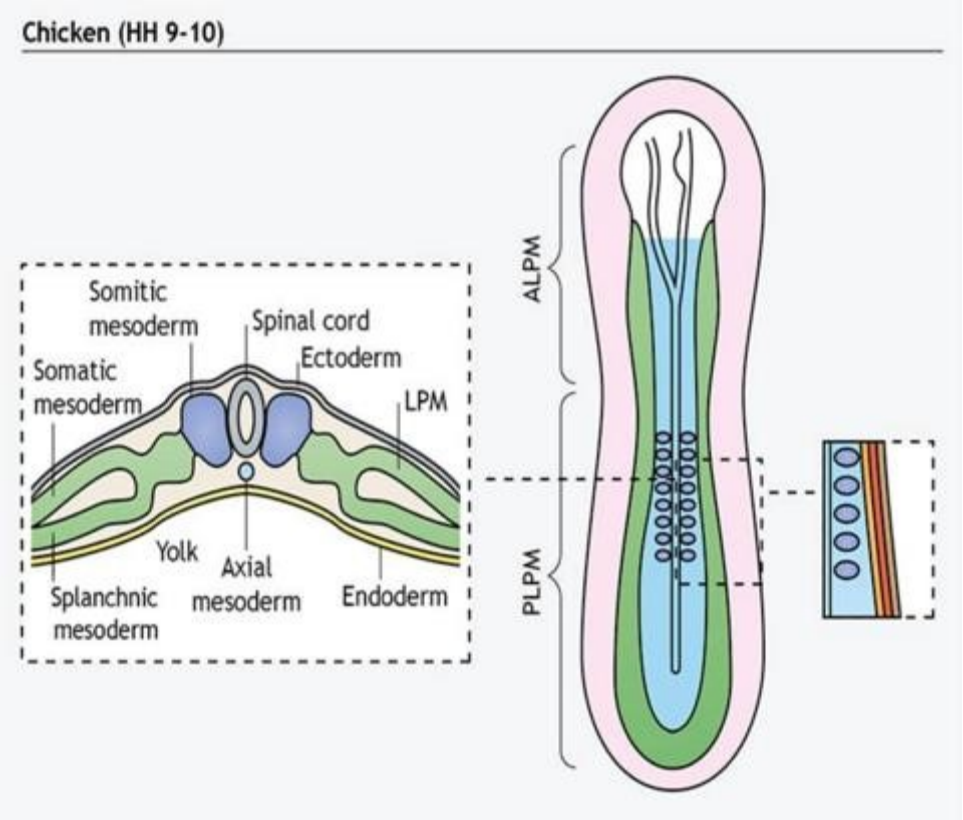

Figure 6- Cross-section and overview of lateral plate mesoderm formation (Prummel, Nieuwenhuize and Mosimann, 2020).

1.4.4 Developmental Potential of the LPM

Despite being morphologically distinct after gastrulation, the LPM remains highly dynamic and challenging to visualise during its earliest stages of formation (Prummel *et al.*, 2020). Lineage tracing and transplantation studies across vertebrate species have demonstrated that the LPM gives rise to a wide array of derivatives, including endothelial and haematopoietic cells, smooth muscle, renal progenitors, and limb connective tissues (Honda, 2021; Prummel *et al.*, 2020). Transplantation and lineage tracing experiments in several species have established that the LPM contains progenitors of the circulatory system, smooth muscles, the kidneys, and limb connective tissue (Prummel et al., 2020; Honda, 2021; Pouncey and Mok, 2025**).** An overview of mesodermal derivatives arising from the LPM and other mesodermal subtypes is shown in Fig. 7.

The specification and differentiation of LPM derivatives are regulated by a network of transcription factors, including *Hand1/2*, *Tbx5*, *Osr1*, *FoxF1*, *Prrx1*, *Mesp1*, and *Etv2*. These factors play overlapping and context-dependent roles in cell fate determination, although their precise functions are not always evolutionarily conserved. How the LPM emerges from an initially heterogeneous mesendodermal population and resolves into distinct mesodermal and endodermal progenitors remains an area of active investigation.

1.4.5 Regulatory Elements and Molecular Markers of the LPM

Several cis-regulatory elements with LPM-specific activity have been identified in vertebrates. These include enhancers upstream of *HoxB6* and *Gata4*, as well as downstream regulatory elements of *Bmp4*. In mice, the *Gata4* LPM enhancer responds to BMP-dependent Smad signalling, consistent with the ventral origin of the LPM (Rojas *et al.*, 2005). However, these enhancers often label restricted posterior LPM domains rather than the entire LPM.

In contrast, studies in zebrafish have identified a 6.35 kb cis-regulatory region of the *draculin (drl)* gene that robustly labels the entire LPM from its emergence during gastrulation through early differentiation (Mosimann *et al.*, 2015; Prummel *et al.*, 2019). Additional transgenic approaches in mouse, using reporters driven by *HoxB6*, *Prrx1*, *Bmp4*, *Gata2*, or *FoxF1*, have enabled visualisation of LPM populations following gastrulation (Prummel *et al.*, 2019).

Together, these studies highlight the central role of the LPM as a multipotent mesodermal population and underscore its importance as the developmental context from which haematoendothelial progenitors arise.

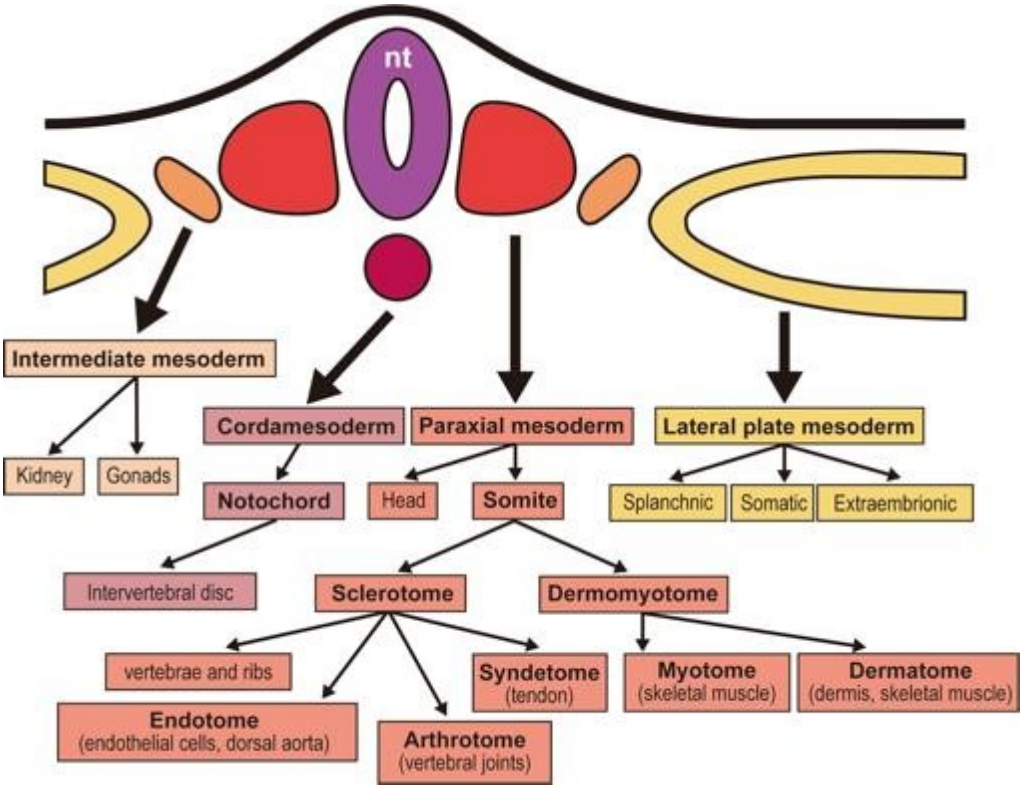

Figure 7- Overview of mesodermal derivatives. (Tani, S., et al, 2020)

2. Hematopoiesis in embryonic development

The establishment of the circulatory system is one of the earliest and most critical events in vertebrate embryogenesis. Blood vessels form prior to the onset of circulation and provide the structural framework that later integrates with the developing heart to enable blood flow (Gilbert, 2017). Closely linked to vascular development is haematopoiesis, the process by which blood cells are generated during embryonic development.

In the chick embryo, blood cells, vascular endothelial cells, and vascular smooth muscle cells arise predominantly from mesodermal populations derived from the lateral plate mesoderm (LPM) and extraembryonic mesoderm. These progenitors originate from ventral mesoderm precursors located in the posterior primitive streak at Hamburger–Hamilton stage HH4 (Alev *et al.*, 2010; Nagai *et al.*, 2018). Lineage-tracing studies have demonstrated that these precursors contribute to both intraembryonic LPM and extraembryonic mesoderm, highlighting their broad developmental potential.

The specification of ventral mesodermal fates is regulated by graded signalling environments within the primitive streak, particularly involving bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Wnt signalling pathways (Nagai *et al.*, 2018). Ventral mesodermal cells undergo epithelial-to-mesenchymal transition (EMT), delaminate from the epiblast, and migrate laterally toward peripheral embryonic and extraembryonic territories, where haematoendothelial development is initiated.

Between HH5 and HH7, ventral mesodermal cells segregate into two principal progenitor populations: hemangioblasts and smooth muscle progenitors (Shin *et al.*, 2009; Nagai *et al.*, 2018). Hemangioblasts are classically defined as bipotential progenitors capable of generating both endothelial and haematopoietic cells (Xiong, 2008). However, clonal analyses indicate that individual hemangioblasts rarely give rise to both lineages *in vivo*, suggesting that lineage commitment often occurs rapidly following specification (Weng *et al.*, 2007). These foundational insights originated from classical chick blastoderm and explant culture studies (Murray, 1932).

Importantly, haematoendothelial progenitors represent a broader developmental category than hemangioblasts. While hemangioblasts are defined functionally by biopotency, HEPs encompass a heterogeneous population of progenitors that contribute to endothelial cells, hemogenic endothelium, and early haematopoietic lineages. Among the endothelial derivatives of HEPs is a specialised transient population known as hemogenic endothelium (Antas *et al.*, 2013). These cells line developing blood vessels and retain the ability to generate blood cells through endothelial-to-haematopoietic transition (EHT) (Gritz and Hirschi, 2016; Ottersbach, 2019). This process gives rise to definitive haematopoietic stem cells (HSCs) during a restricted developmental window (Fig. 8).

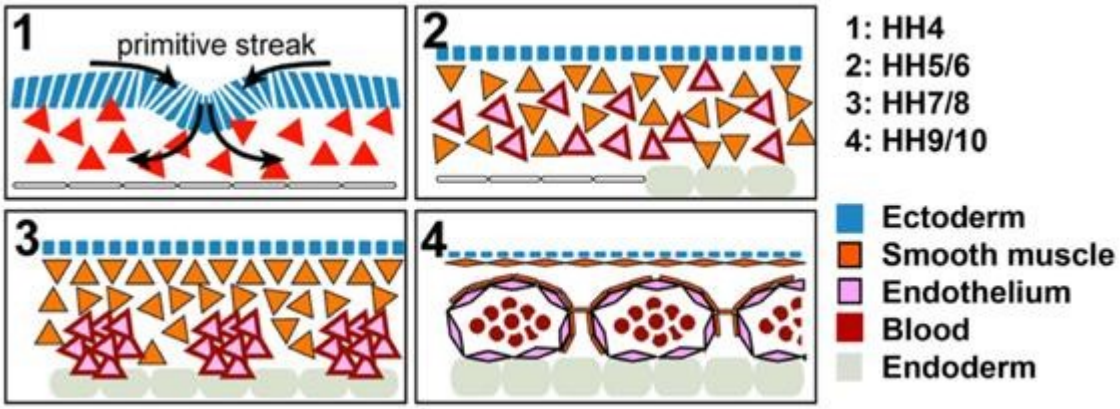

Figure 8- Cellular morphogenesis in early hematopoietic and vascular development in chick (Nagai et al., 2018)

2.2 Blood island formation

The field of hematopoietic and vascular developmental research owes its origin to the chick embryo (Nagai et al., 2018; Pouncey and Mok, 2025). The earliest morphological indication of haematopoietic differentiation in the chick embryo is the formation of blood islands within the extraembryonic mesoderm of the yolk sac. At Hamburger–Hamilton stages HH5–HH6, migrating hemangioblasts aggregate into discrete clusters known as blood islands, marking the onset of primitive haematopoiesis (Cumano and Godin, 2007). Each blood island represents a functional unit in which endothelial and haematopoietic lineages differentiate in close spatial association.

By HH7, cells within blood islands begin to diverge into distinct fates. Centrally located cells differentiate toward a haematopoietic lineage and express erythroid markers, including embryonic haemoglobins, while peripheral cells adopt an endothelial fate and contribute to the surrounding vascular network. Hemangioblast-derived progenitors express transcription factors such as *Scl* and *Lmo2*, whereas neighbouring smooth muscle and mesothelial progenitor's express markers such as *Hand2* (Xiong, 2008; Nagai *et al.*, 2018).

As development progresses to HH8–HH9, regional differences emerge in blood island fate. Smaller blood islands within the embryonic lateral plate mesoderm and proximal extraembryonic regions do not produce blood cells but instead undergo endothelial differentiation and morphogenesis. These endothelial populations contribute to vascular connections between the developing endocardium and the expanding extraembryonic vascular plexus (Nagai *et al.*, 2018).

By HH9–HH11, the extraembryonic vascular plexus becomes contiguous with the developing endocardium anteriorly and the paired dorsal aortae posteriorly. This interconnected network subsequently gives rise to the vitelline veins and arteries, establishing a functional embryonic–extraembryonic circulatory interface (Le Noble *et al.*, 2004; Seco *et al.*, 2020).

Primitive haematopoietic cells generated within blood islands are thought to arise through two related mechanisms: direct differentiation from hemangioblasts or via an intermediate hemogenic

endothelial state. These alternative models of blood island development are illustrated in Fig. 9 (Cumano and Godin, 2007).

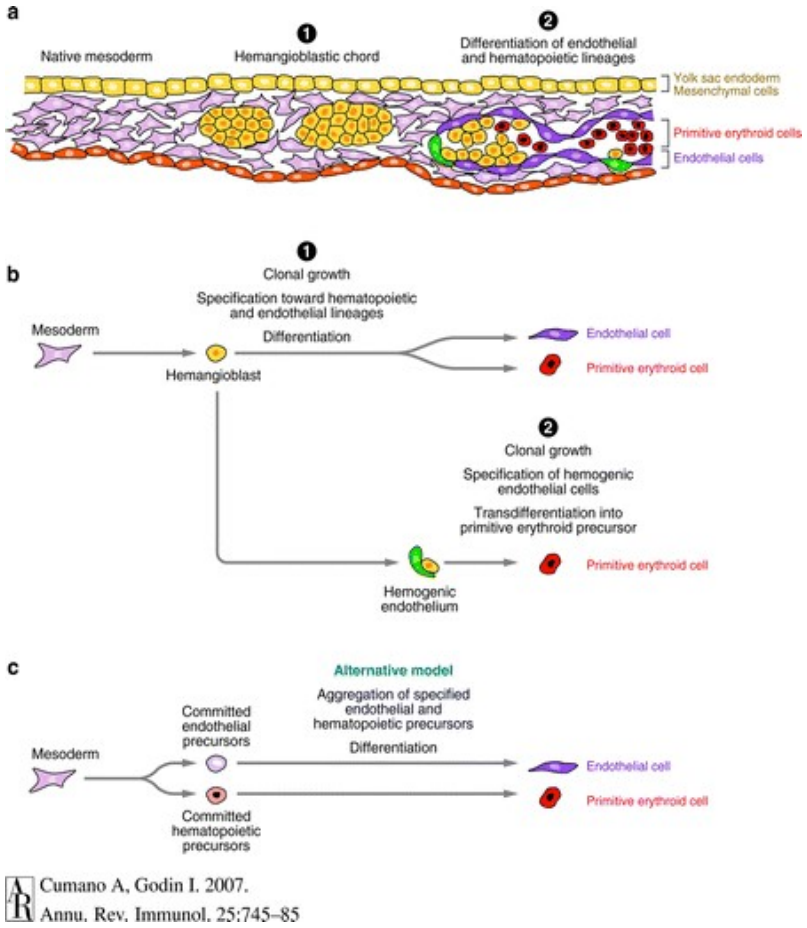

Figure 9- Mechanism of yolk sac (YS) blood island formation. (a) Schematic representation of the progressive evolution of blood island mesodermal cells to a functional vascular network and primitive erythroid cells. Two alternative mechanism of YS blood island formation are shown. (b) Endothelial and hematopoietic cells may be generated directly from progenitors restricted to both differentiation pathways, the hemangioblast, or via the intermediate production of hemogenic endothelial cells. (c) Alternatively, the specification into endothelial and hematopoietic cells might occur at earlier stages of differentiation (c) (Cumano and Godin, 2007).

As the vascular plexus forms by stage HH10, blood cells start to lose direct contact with each other, indicating they are preparing to circulate. The heart begins to function by stages HH12-HH13, and the vascular system is fully formed, allowing blood cells to circulate freely. During this stage, mesothelial precursors from the ventral mesoderm differentiate into layers that contribute to the lining of the body cavities and blood vessels (Nagai et al. (2018).

Therefore, the blood islands in the yolk sac (Figure 8) play a pivotal role in forming the extraembryonic vascular network and are responsible for generating the initial hematopoietic cells in the developing embryo. Much of our understanding of how blood islands form is derived from studies conducted in chick embryos. The hemangioblast requires yolk sac visceral endoderm-derived factors for blood island formation. The blood islands that line the yolk sac produce the vitelline veins that that bring the nutrients to the embryo and transport gases to and from the sites of respiratory exchange. Figure 11 further depicts the Hemangioblastic origin of aortic hemogenic endothelial cells. In chick embryos, hemangioblasts emerge in the yolk sac during Hamburger and Hamilton stage (HH) stage 5-6, aggregating into blood islands at HH7-8.

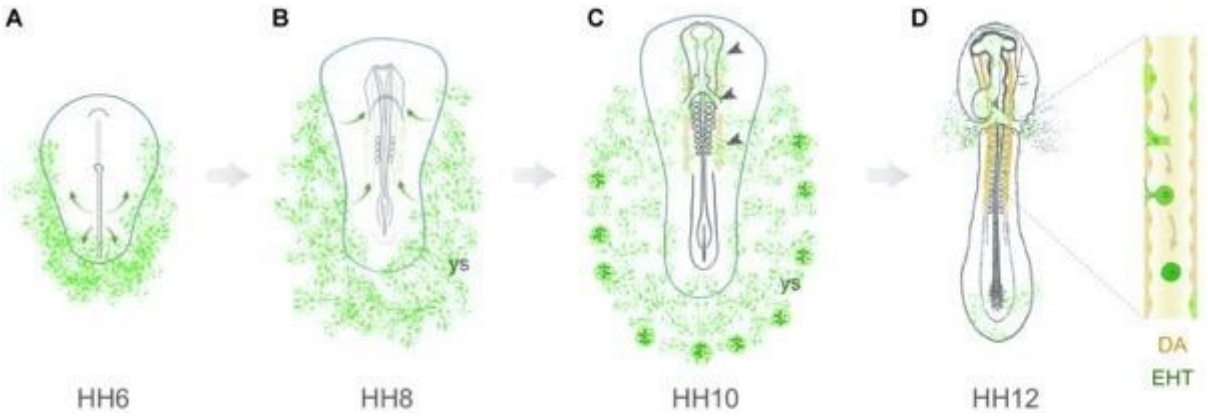

Figure 10- Hemangioblastic origin of aortic hemogenic endothelial cells in the early chick embryo. (A) hemangioblasts arise from lateral plate mesodermal cells that ingress through the posterior region of the primitive streak. HH6, cells are in the yolk sac and intraembryonic region. (B) HH7-8, hemangioblasts aggregate to form blood islands within the yolk sac (ys) hemangioblast- derived cells start to migrate toward the presumptive paired dorsal aortae (orange). (C) HH10, yolk sac hemangioblasts have already started to be differentiated into endothelial and hematopoietic cells in the distal blood islands. (D) HH11-12, hemangioblast-derived HECs are detected in the dorsal aortae (DA), where they undergo an endothelial-to hematopoietic transition EHT (Seco et al., 2020).

2.3 Experimental evidence and historical perspective on hemangioblasts

The concept of the hemangioblast emerged from the observation that blood and endothelial cells arise in close spatial and temporal association during early embryogenesis, particularly within yolk sac blood islands. Initially proposed as a theoretical common precursor, the hemangioblast hypothesis gained support from experimental studies demonstrating shared developmental origins and overlapping molecular programs between early endothelial and haematopoietic lineages.

Evidence for a common haematoendothelial precursor has been obtained in several vertebrate systems using clonal analyses and lineage-tracing approaches, which indicate that mesodermal progenitors can contribute to both endothelial and haematopoietic lineages *in vivo* (C. Biben et al., 2023). Additional support for this model comes from the finding that early endothelial and haematopoietic populations share the expression of multiple genes associated with haematoendothelial specification, consistent with a shared developmental programme.

Genetic evidence from mouse models also supports a close developmental relationship between endothelial and haematopoietic lineages. For example, gene targeting experiments disrupting the receptor tyrosine kinase Flk1 (VEGFR2/Kdr), a key regulator of vascular development, result in severe defects in both endothelial and blood lineages, consistent with an early requirement for Flk1-positive progenitors in haematoendothelial development. In vitro studies further strengthen this model. In avian embryos, Flk1-positive cells isolated from the early blastodisc can generate endothelial colonies in the presence of vascular endothelial growth factor (VEGF), while alternative culture conditions support the formation of haematopoietic colonies. Similarly, in mouse embryonic stem cell differentiation cultures, the blast colony-forming cell (BL-CFC) has been identified as a candidate hemangioblast population that can produce both endothelial and haematopoietic lineages in response to VEGF and stem cell factor (SCF).

While these findings support the existence of progenitors with dual haematoendothelial potential, it is important to distinguish the hemangioblast concept from haematoendothelial progenitor (HEP) cells discussed earlier. Hemangioblasts are classically defined as bipotential progenitors, whereas HEPs represent a broader, heterogeneous group of progenitors that may include bipotential cells, lineage-restricted progenitors, and endothelial intermediates such as hemogenic endothelium. Thus, hemangioblasts and HEPs are related concepts, but they are not strictly synonymous.

A key intraembryonic site where the endothelial–haematopoietic relationship becomes especially clear is the dorsal aorta. The dorsal aorta is the first major intraembryonic blood vessel to form and is also a crucial site of secondary (definitive) haematopoiesis (Seco *et al.*, 2020). In avian embryos, angioblasts derived from the splanchnic mesoderm assemble into paired longitudinal endothelial cords that subsequently remodel and fuse to form the dorsal aorta. During this process, haematoendothelial progenitors emerge within the aortic endothelium, supported by signals from neighbouring tissues and the incorporation of somite-derived endothelial contributions. Importantly, the dorsal aorta functions not only as a conduit for blood flow but also as a specialised microenvironment that promotes haemogenic endothelial specification and the subsequent emergence of haematopoietic cells (Seco *et al.*, 2020).

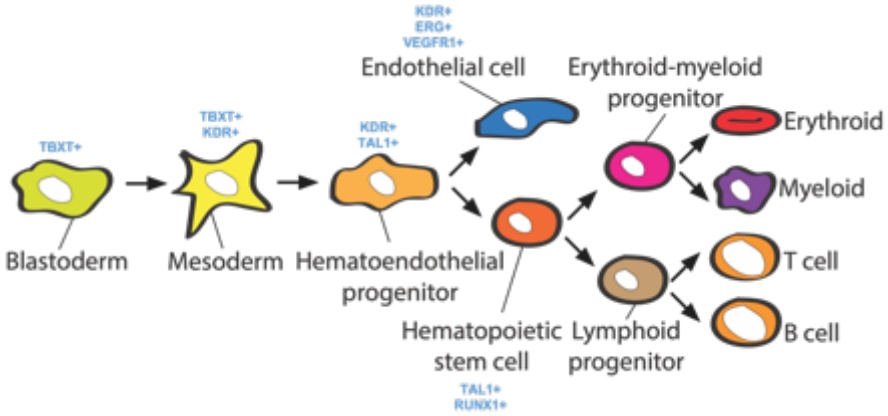

Figure 11. Developmental relationships between endothelial and haematopoietic lineages.

2.4 Epithelial-to-Hematopoietic transition (EHT)

A key mechanism linking vascular and haematopoietic development during embryogenesis is the endothelial-to-haematopoietic transition (EHT)**(**Pouncey and Mok, 2025)**.** During this process, specialised endothelial cells known as hemogenic endothelium give rise to haematopoietic progenitor cells through a tightly regulated trans differentiation event. EHT represents a critical developmental step in which endothelial identity is progressively lost and a haematopoietic transcriptional programme is activated.

Following their initial specification, haematoendothelial progenitors (HEPs) and early haematopoietic progenitor cells migrate from sites of primitive haematopoiesis, such as the yolk sac blood islands, toward regions that support further maturation and lineage diversification (Ottersbach, 2019). This migration is guided by chemotactic cues and local signalling environments that ensure colonisation of permissive haematopoietic niches.

One of the most important sites of definitive haematopoiesis in vertebrate embryos is the aorta–gonad–mesonephros (AGM) region. In avian embryos, as in mammals, the AGM encompasses the dorsal aorta, gonadal primordia, and mesonephros and provides a specialised microenvironment that supports haematopoietic development. Within this region, the dorsal aorta plays a central role as a transient haematopoietic organ (de Bruijn, 2000).

During early development, hemogenic endothelial cells lining the ventral wall of the dorsal aorta acquire haematopoietic potential and undergo EHT. Morphologically, this process is characterised by endothelial cells rounding up, losing their tight junctions and apical–basal polarity, and budding into the aortic lumen as nascent haematopoietic cells. These cells subsequently detach and enter circulation or seed secondary haematopoietic organs. The dorsal aortic endothelium therefore functions not only as a structural component of the vasculature but also as a dynamic niche that promotes haematopoietic specification.

EHT is regulated by a combination of intrinsic transcriptional changes and extrinsic signals derived from the surrounding microenvironment. Signalling inputs from neighbouring tissues, including the Somites and mesenchyme, as well as haemodynamic forces generated by the onset of blood flow, contribute to the induction and regulation of hemogenic endothelium. This

tightly controlled process ensures that haematopoietic progenitors are generated within a restricted temporal window and in defined anatomical locations.

During chick development, haematopoiesis occurs in successive waves, each characterised by distinct sites of origin, progenitor types, and developmental timing (de Bruijn, 2000; Taylor, Taoudi and Medvinsky, 2010). EHT is a defining feature of definitive haematopoiesis and is essential for the generation of multipotent haematopoietic stem cells capable of long-term self-renewal and multilineage differentiation.

Together, these findings highlight EHT as a fundamental developmental mechanism that integrates vascular morphogenesis with blood cell formation and underscores the close developmental relationship between endothelial and haematopoietic lineages.

2.5 Primitive Haematopoiesis

Primitive haematopoiesis represents the first wave of blood cell formation during embryonic development and occurs predominantly in the extraembryonic yolk sac. This early haematopoietic programme is responsible for generating a transient population of blood cells that support the immediate physiological needs of the developing embryo, particularly oxygen transport.

In chick embryos, primitive haematopoiesis initiates at approximately Hamburger–Hamilton stages HH5–HH6 within the yolk sac blood islands. This wave primarily produces nucleated, embryonic haemoglobin–expressing erythrocytes, which provide oxygen to rapidly growing embryonic tissues before the establishment of a fully functional vascular system. These early erythroid cells are morphologically and transcriptionally distinct from blood cells generated during later developmental stages.

Comparative studies in mammals indicate that primitive haematopoiesis begins around embryonic day E7.25 in the yolk sac blood islands. In addition to primitive erythrocytes, this wave also produces macrophages and megakaryocyte progenitors but does not generate self-renewing haematopoietic stem cells (HSCs) (Palis, 2014; Tober *et al.*, 2007). Primitive erythrocytes are larger than their definitive counterparts, retain their nuclei, and express embryonic globin genes, reflecting their specialised and temporary role. Importantly, progenitors generated during primitive haematopoiesis lack long-term self-renewal capacity and multilineage potential, distinguishing this wave from definitive haematopoiesis.

Primitive haematopoiesis is therefore a transient but essential developmental programme that ensures early oxygen delivery and innate immune support during embryogenesis, while subsequent haematopoietic waves generate the long-term blood system.

2.6 Genes involved in primitive haematopoiesis

The specification of blood cell fates during primitive haematopoiesis is governed by a core transcriptional network, with the transcription factors GATA1 and PU.1 (SPI1) playing central and antagonistic roles in directing erythroid and myeloid lineage commitment.

GATA1 is a key regulator of erythroid differentiation and is required for the proper maturation of primitive erythroid, megakaryocytic, mast cell, and eosinophil lineages. In the early mouse

embryo, loss of *Gata1* results in severe defects in primitive erythropoiesis, characterised by impaired differentiation and reduced survival of erythroid progenitors (Fujiwara *et al.*, 2004). As a result, GATA1 is widely regarded as a master regulator of erythrocyte development (Cantor and Orkin, 2002).

Studies in zebrafish further support the conserved role of Gata1 during primitive haematopoiesis. *gata1*-expressing cells in zebrafish embryos express erythrocyte-specific haemoglobins, as demonstrated by benzidine staining, indicating active embryonic globin gene expression. In addition to promoting erythroid gene expression, Gata1 actively suppresses myeloid differentiation. Knockdown of *gata1* in zebrafish results in a lineage switch, whereby presumptive erythroid cells adopt a myeloid fate and upregulate myeloid-specific genes such as *pu.1* and *mpo*.

PU.1, encoded by *Spi1*, is a master regulator of myeloid lineage specification, including macrophage and granulocyte development. Functional studies demonstrate that PU.1 and GATA1 engage in a cross-inhibitory regulatory relationship, ensuring mutually exclusive lineage commitment during primitive haematopoiesis. Consistent with this model, knockdown of *pu.1* in zebrafish embryos leads to increased *gata1* expression within the anterior lateral mesoderm and promotes erythroid differentiation, as evidenced by the expression of erythroid markers such as *hbae1* (Rhodes *et al.*, 2005).

Together, these findings highlight a conserved transcriptional switch in which the balance between GATA1 and PU.1 activity determines primitive erythroid versus myeloid cell fate. This tightly regulated antagonism ensures appropriate lineage allocation during the earliest wave of haematopoiesis and provides a mechanistic framework for understanding how early blood cell diversity is generated prior to the emergence of definitive haematopoietic stem cells.

2.7. Definitive Haematopoiesis

Definitive haematopoiesis represents the second major wave of blood cell formation during embryonic development and is distinguished from primitive haematopoiesis by the generation of multipotent, self-renewing haematopoietic stem cells (HSCs). These HSCs give rise to all mature blood lineages and sustain haematopoiesis throughout postnatal life.

In chick embryos, definitive haematopoiesis begins around Hamburger–Hamilton stages HH9–HH10 and is marked by the emergence of erythroid–myeloid progenitors (EMPs), which generate adult-type erythrocytes and myeloid cells with increased functional specialisation. Unlike primitive haematopoietic cells, definitive progenitors exhibit broader lineage potential and contribute to long-term blood production.

Across vertebrates, definitive haematopoiesis occurs at multiple anatomical sites in a temporally regulated manner. Following their emergence, haematopoietic progenitors colonise secondary haematopoietic organs where they undergo expansion and maturation. In amniotes, including the chick, these sites include the aorta–gonad–mesonephros (AGM) region, the fetal liver, and later the spleen and bone marrow. In the post-hatching chick, the bone marrow becomes the principal site of haematopoiesis and remains responsible for blood cell production throughout adult life (Jagannathan-Bogdan and Zon, 2013).

The transition from primitive to definitive haematopoiesis reflects a fundamental developmental shift in both the cellular properties and regulatory mechanisms governing blood formation, with definitive haematopoiesis requiring specialised vascular niches and distinct transcriptional control.

2.8 Genes involved in definitive haematopoiesis

A key regulator of definitive haematopoiesis is the transcription factor RUNX1, a member of the Runt-related family of transcription factors. RUNX1 is essential for the initiation of the definitive haematopoietic programme and plays a central role in enabling the endothelial-to-haematopoietic transition (EHT). Genetic studies in mice demonstrate that *Runx1* is indispensable for definitive erythroid, myeloid, and lymphoid development, as *Runx1*-null embryos completely lack these lineages (Bertrand *et al.*, 2005).

In zebrafish, *runx1* expression is first detected at the five-somite stage in the posterior lateral plate mesoderm and subsequently becomes localised to the dorsal aorta, coinciding with the emergence of haemogenic endothelium. Functional disruption of *runx1* impairs definitive, but not primitive, haematopoiesis, confirming its specific role in the generation of long-term haematopoietic stem and progenitor cells (Rhodes *et al.*, 2005).

At the molecular level, RUNX1 acts as a master regulator by activating haematopoietic gene expression programmes and repressing endothelial identity within hemogenic endothelial cells. This transcriptional switch is a defining feature of definitive haematopoiesis and distinguishes it from earlier blood-forming waves. RUNX1 therefore serves as a molecular hallmark of definitive haematopoietic specification and HSC emergence.

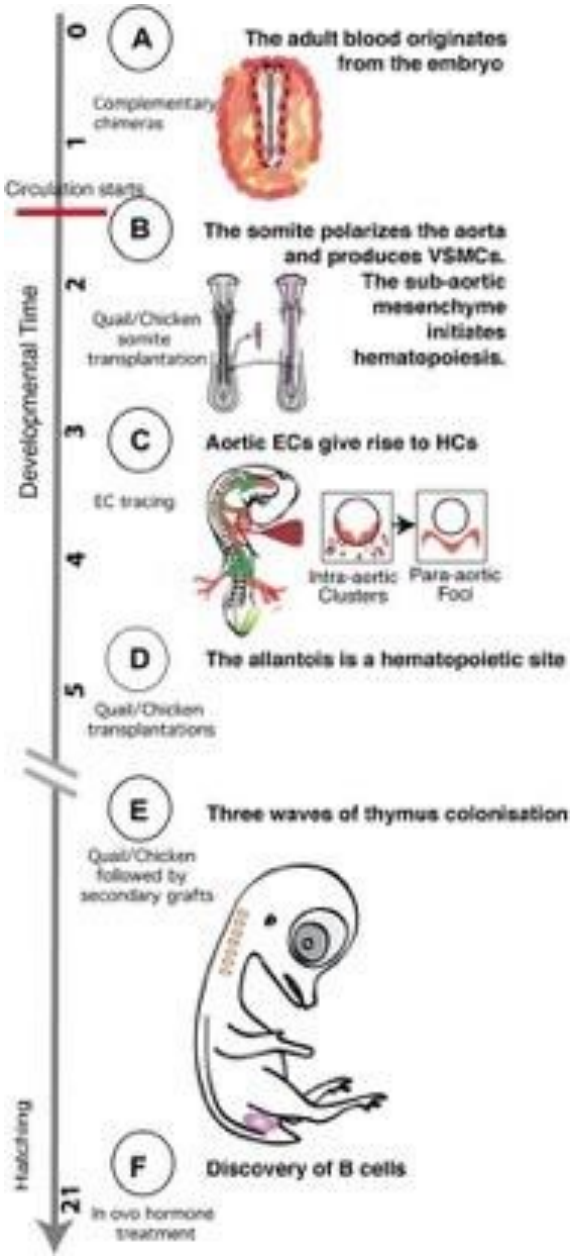

Figure 12- Chicken development timetable and some of the major discoveries the avian model has contributed to the hematopoietic field. The scale on the left indicates developmental time (Jaffredo and Yvernogeau, 2014).

2.9 Aorta Gonad Mesonephros (AGM)

The aorta–gonad–mesonephros (AGM) region is the primary intraembryonic site of definitive haematopoietic stem cell emergence during vertebrate development. In mammals, definitive HSCs first appear in the AGM at approximately embryonic day E10.5 (Medvinsky and Dzierzak, 1996; Müller *et al.*, 1994), while comparable stages have been identified in avian embryos. The AGM arises from mesodermal tissue and comprises three anatomically distinct structures: the dorsal aorta, the genital ridges, and the mesonephros.

Definitive HSCs originate from specialised hemogenic endothelial cells lining the dorsal aorta through endothelial-to-haematopoietic transition (EHT). During this process, hemogenic endothelial cells upregulate *Runx1*, lose endothelial characteristics, and bud into the aortic lumen as haematopoietic clusters (Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Jaffredo *et al.*, 1998). These intra-aortic haematopoietic clusters are a hallmark of definitive haematopoiesis and are conserved across vertebrate species.

Although most haematopoietic clusters emerge from the ventral wall of the dorsal aorta, minor contributions from the dorsal endothelium have also been reported. Following their emergence, AGM-derived HSCs enter circulation and migrate to secondary haematopoietic organs, most notably the fetal liver, where they undergo extensive expansion and maturation (Crisan and Dzierzak, 2016). Later in development, HSCs colonise the bone marrow, which becomes the lifelong site of haematopoiesis.

Interestingly, functional HSCs have also been detected in the vitelline and umbilical arteries during this developmental window, suggesting that definitive haematopoiesis is not restricted exclusively to the AGM but may arise from a broader intraembryonic vascular domain (de Bruijn *et al.*, 2001). In humans, definitive HSCs emerge at approximately 4–5 weeks of gestation and can be identified by their capacity to reconstitute haematopoiesis in immunocompromised mouse models.

The emergence and maturation of HSCs within the AGM is tightly regulated by the local microenvironment, including signals from surrounding tissues, haemodynamic forces, and components of the extracellular matrix. Increasing evidence suggests that this niche plays an active role in directing haemogenic endothelial specification and HSC fate decisions, highlighting the importance of the AGM as both a structural and signalling hub for definitive haematopoiesis.

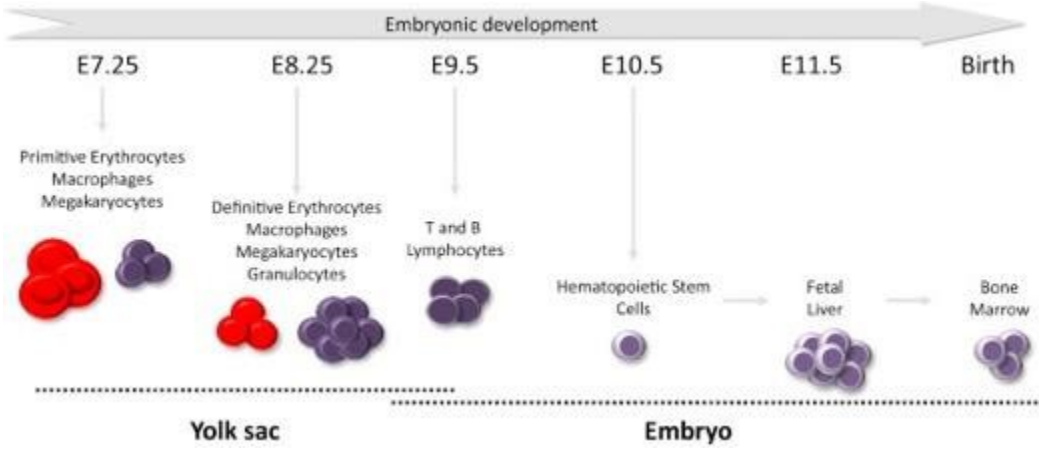

Figure 13- Sequential waves of embryonic haematopoiesis. Shown is a schematic representation of hematopoietic development during embryogenesis with the embryonic day of emergence, the type of progenitors generated, and the localization of each wave ***(Lacaud and Kouskoff, 2017)***

3. The Extracellular Matrix

Our understanding of ECM composition, structure and function has grown considerably in the last several decades and this knowledge has revealed that the extracellular matrix is important for cell growth, survival, differentiation, and morphogenesis. The ECM surrounds all mammalian cells, forming a distinct pericellular matrix in some cell types. It is made up of several components that bond to form a complex network of different-sized molecules in a 3D unit. These ECM molecules are of different sizes, shapes, and spatial organization.

The complex organisation of the niche ECM comprises mainly of macromolecules such as collagens, glycosaminoglycans, proteoglycans, elastin, laminins, fibronectin and other proteins such as matricellular proteins.

Once synthesized in the cytoplasm, the ECM components are secreted into the extracellular space where they are then modified further into final molecules. The main recognized function of the ECM is the provision of physical support for cells within tissues and organs as well as availing the transportation of biomolecules such as growth factors and cytokines to cells. Furthermore, the ECM niche can be considered a reservoir where proteins bind to proteoglycans for signal delivery.

Multiple ECM proteins are involved in HSC differentiation, lineage specification, proliferation, and apoptosis. This section will go over the ECM structure and composition in detail and explore its role in haematopoiesis and cardiac development.

3.1 Functions of the ECM

The extracellular matrix (ECM) is a dynamic extracellular network produced and remodelled by cells. Rather than acting solely as a passive scaffold, the ECM provides biochemical and mechanical cues that influence key cellular behaviours, including proliferation, migration, adhesion, polarity, and differentiation. In developing embryos, these ECM-derived cues work alongside intrinsic gene regulatory programmes to shape tissue morphogenesis and lineage specification.

ECM composition is tissue-specific and is determined by differential expression of ECM genes, alternative splicing, and extensive post-translational modifications (Mead, 2018). Importantly, cell–ECM interactions are spatially organised. For example, epithelial and endothelial cells primarily contact basement membrane components on their basal surfaces, while mesenchymal cells interact predominantly with interstitial ECM. Distinct cell–ECM junctions also support these interactions, including hemidesmosomes in epithelial cells and integrin-based adhesions in mesenchymal cells. Through these interfaces, the ECM can modulate signalling pathways and tune cellular behaviours in a context-dependent manner.

During development, the ECM can regulate multiple processes simultaneously acting as a scaffold, a reservoir for growth factors, and a mechanical substrate that influences cell shape and movement. Figure 14 summarises broad ECM functions that are particularly relevant during embryogenesis, including morphogenesis, migration, tissue boundary formation, and lineage specification (Fig. 14).

Large-scale ‘omics’ studies have grouped ECM components into the “matrisome,” which includes core structural ECM proteins as well as ECM-associated regulators and secreted factors (Dzobo and Dandara, 2023). Together, these macromolecules form a dynamic network that actively shapes tissue architecture and developmental decision-making.

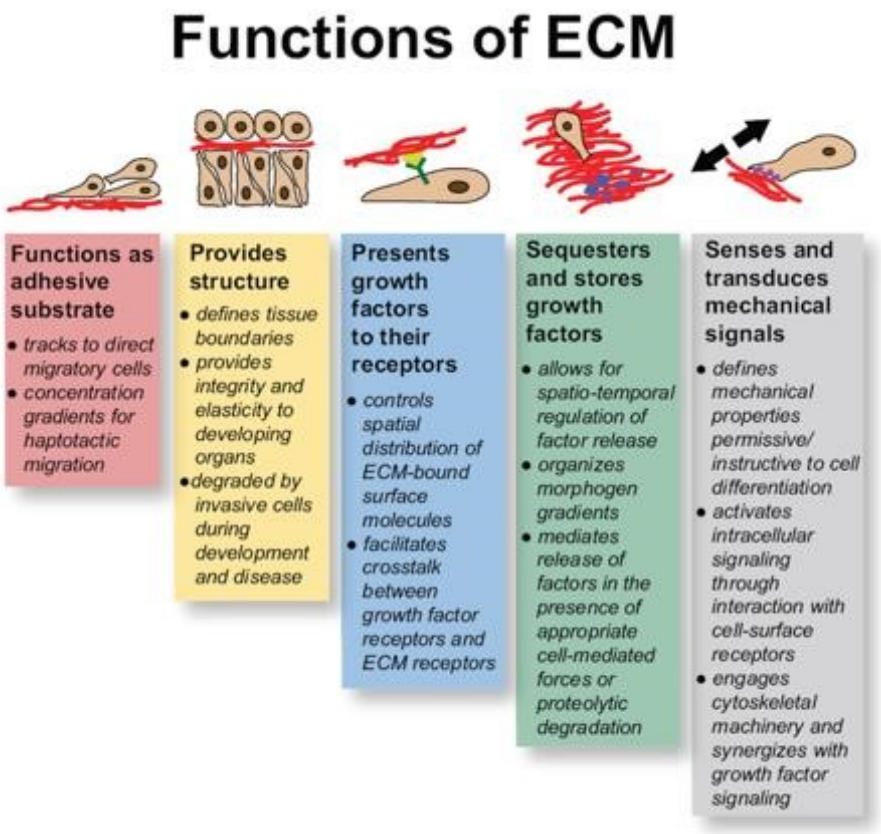

Figure 14- Summary of ECM functions in development.
The ECM can influence multiple biochemical and mechanical processes simultaneously. This figure illustrates different functional states of the ECM and their biological contexts. The five categories are not mutually exclusive.

Comprehensive 'omics' studies have classified many ECM components collectively as the 'matrisome', with more than 200 ECM- related genes identified in humans (Dzobo and Dandara, 2023). These macromolecules form a fibrillar network that interacts dynamically with cells and biomolecules to influence tissue architecture and cell behaviour.

3.2. The two compartments of ECM: Basement membrane and interstitial membrane

The ECM can be broadly organised into two interconnected compartments: the basement membrane (BM) and the interstitial matrix (IM) (Fig. 15). The basement membrane is a thin, specialised ECM layer underlying epithelial and endothelial cells, whereas the interstitial matrix occupies the extracellular space between stromal and connective tissue cells.

The basement membrane typically provides structural support, establishes cell polarity, and regulates selective permeability and signalling. In contrast, the interstitial matrix forms a more fibrillar, three-dimensional meshwork that contributes to tissue mechanics and provides migration substrates. Although the BM and IM are compositionally distinct, they function as a coordinated ECM system that is continuously remodelled during development and disease (Dzobo and Dandara, 2023).

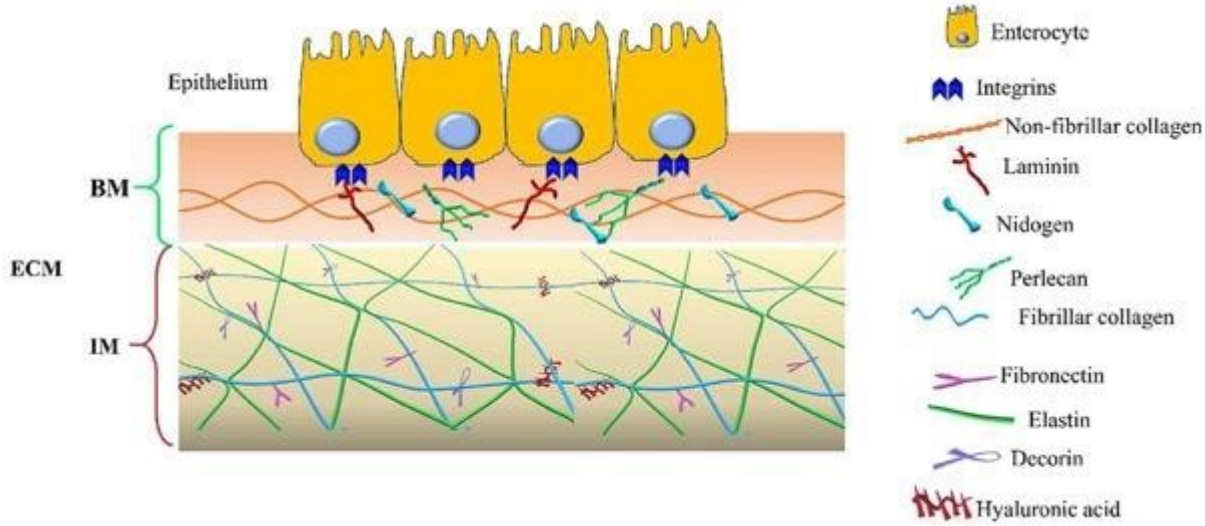

Figure 15- Schematic of the Extracellular matrix. Schematic representation of the main components of the two ECM compartments: basement membrane (BM) and interstitial matrix (IM). The legend indicates the identity of each ECM components.

3.2.1 The Basement membrane (BM)

The basement membrane is a specialised ECM sheet (typically ~50–100 nm thick) located between epithelial/endothelial layers and underlying mesenchyme. It contains a conserved core set of components, most notably laminins, collagen IV, nidogens, and heparan sulphate proteoglycans such as perlecan (HSPG2). Laminins and collagen IV self-assemble into networks that provide structural stability, while nidogens and perlecan contribute to crosslinking and growth factor binding. Through these interactions, the BM provides both mechanical integrity and signalling regulation.

Perlecan is particularly relevant in vascular contexts because it can bind growth factors such as FGF and VEGF and thereby modulate angiogenic signalling. Consistent with this, basement membranes are prominent in blood vessels as well as epithelial tissues (Tanzer, 2006).

3.2.2 Interstitial matrix (IM)

The interstitial matrix lies beneath the basement membrane and forms a major structural component of connective tissues. It contains fibrillar collagens, elastin, fibronectin, and proteoglycans, producing a hydrated 3D matrix whose composition varies by tissue type and developmental stage (Frantz *et al.*, 2010). In many contexts, fibronectin plays a key organisational role by guiding matrix assembly and providing integrin-mediated adhesion sites, thereby influencing migration and morphogenesis.

Communication between cells and the ECM is also important to understanding how the ECM and cells learn to adapt to each other.

3.3. Components of the ECM

The most relevant ECM components are those that regulate cell migration, adhesion, polarity, and signalling during early development and haematoendothelial specification. Fibronectin is a major organiser of early embryonic ECM and a crucial substrate for morphogenetic migration; fibronectin loss-of-function phenotypes often include severe gastrulation and cardiovascular defects (Rozario and DeSimone, 2010).

Laminins and collagen IV (basement membrane) provide structural stability and signalling platforms for epithelial/endothelial tissues, influence polarity and barrier functions.

Proteoglycans and glycosaminoglycans (e.g., heparan sulphate, hyaluronan) regulate hydration, tissue mechanics, and growth factor availability; can localise cytokines and morphogens to shape developmental signalling landscapes (Toole, 2001; Camenisch *et al.*, 2000; Spicer *et al.*, 2002; Chen *et al.*, 2007).

Together, these ECM systems function as both scaffolds and signalling regulators, supporting the hypothesis that ECM composition and organisation can influence haematoendothelial progenitor behaviour and lineage commitment.

3.4. The ECM in the early embryo

In many embryos, the ECM is synthesized and assembled just prior to or coincident with the initiation of gastrulation movements. The ECM possesses a complex macro molecular structure requiring many interacting proteins for their assemble and function making the elucidation of the ECM and the ECM receptor functions during gastrulation challenging (Walma & Yamada, 2020). The interactive nature of the ECM is of particular interest in embryos, where the ECM molecules help to direct cellular and epithelial movements and to localize inductive signals (Walma & Yamada, 2020).

Studies have shown that during the early stages of development, the ECM moves at roughly the same speed as the overlaying epiblast cells. This suggests that the epiblast cells are not moving actively, are carried by the ECM, or the epiblast cells have to get traction from their neighbours for all the observed movements.

It has been shown that, during the early stages of development, the extracellular matrix moves at roughly the same speed as the overlaying epiblast cells (Chuai, M et al., 2012) It therefore appears likely that the epiblast cells cannot get any traction from this matrix for their movements. This either implies that the epiblast cells are not moving actively, they are carried

by the extracellular matrix, or the epiblast cells have to get traction from their neighbours for all the observed movements (Chuai M et al., 2012)

Cells deposit extracellular matrix molecules at very early stages of embryonic development. The chick embryo at stage X (blastoderm, Eyal-Giladi and Kochav, 1976) is a flat compacted disc and is homologous to late morula (Eyal-Giladi, 1995).

Deposition of extracellular matrix was first detected in the space between the epiblast and the hypoblast (homologous to blastocoele in amphibia) in embryos at the blastula stage. A great number of doughnut-like granules were accumulating in the blastocoele, and these granules seemed to originate from both the epiblast and the hypoblast. The extracellular matrix was assembled as a fibrillar network in the blastocoele preceding the extensive cellular migrations of gastrulation During gastrulation, cells move as a stream of loosely packed cells away from the primitive streak (homologous to the blastopore in amphibia) in the blastocoele between the epiblast and the hypoblast to form the definitive endoderm and mesoderm; migrating cells interact directly with the matrix through their filopodia that contact and follow gold labelled laminin-containing fibrils (Zagis, Stavridis and Chung (2011).

Gold particles are bound at regular intervals along laminin-containing fibrils in extracellular matrix; laminin is also detected on the cell surface of the migrating cells and in the epiblast and hypoblast. These results indicated a possible direct interaction between cell processes and laminin during active migration and that laminin was involved in providing the substrate and cues for the earliest morphogenetic movements.

The table below focused on summarizing ECM loss-of-function phenotypes in the developing embryo including the ECM genes involved in my investigation.

ECM Component	**Isoform**	**Loss-of-Function Phenotypes**
Fibronectin		Embryonic lethal (~E10.5). Cardia bifida, defects in mesoderm specification, axis elongation, neural tube morphogenesis, myocardial precursor migration, and yolk sac vasculature.
Laminin	α1	Embryonic lethal (E6.5). Extraembryonic tissue developmental defects, epiblast polarization defects, compromised parietal and visceral endoderm differentiation, induction of apoptosis, axis elongation, and eye defects.
	α2	Post-natal death (5 weeks). Muscular dystrophy.
	α3	Post-natal death (3 days). Severe skin blistering.
	α4	Viable (small increase in deaths after birth). Defects in synaptic specialization, haemorrhages, cardiovascular defects.

	α5	Embryonic lethal (before E17). Exencephaly, syndactyly, extraembryonic tissue disorganization, and fin formation defects.
	β1	Embryonic lethal (E5.5). Defects in extraembryonic tissue development, gastrulation, implantation, notochord differentiation, and eye formation.
	β2	Post-natal death (15–30 days). Growth arrest, neuromuscular junction, and renal defects.
	β3	Death just after birth. Severe skin blistering.
	γ1	Embryonic lethal. Notochord differentiation and eye defects.
	γ2	Post-natal death (5 days). Severe skin blistering.
Collagen	ColI	Embryonic lethal (E12–14). Aortic rupture and severe tissue integrity defects.
	ColII	Death at birth. Cartilage formation defects.
	ColIII	Post-natal death (2 days). Growth retardation, reduced life span, skin blistering, blood vessel rupture.
	ColIV	Embryonic lethal (E10.5–11.5). Defects in basement membrane integrity, Reichert's membrane integrity, and growth retardation. Renal failure (Col4α3/α4/α5).
	ColV	Embryonic lethal (E10). Collagen fibril assembly defects, compromised skin, and connective tissue integrity.
	ColVI	Viable. Joint degeneration, musculoskeletal abnormalities, lower body weight, decreased bone density.
	ColVII	Post-natal death (2 weeks). Cutaneous blistering.
	ColVIII	Viable. Notochord and eye defects.
	ColIX	Viable. Non-inflammatory degenerative joint disease, cartilage maintenance defects, abnormal fin vascular plexus development.
	ColX	Viable. Defects in growth plate development, trabecular morphology, bone architecture, and craniofacial skeleton.
	ColXI	Death at birth. Compromised chondrocyte differentiation, severe cartilage defects in limbs, ribs, mandible, and trachea.
	ColXII	Viable. Periodontal ligament and skin matrix architecture abnormalities (not null).
	ColXIV	Viable. Defects in fiber and fibril assembly in tendons.

	ColXV	Viable. Mild muscular and cardiovascular defects, compromised notochord and somite differentiation.
	ColXVII	Post-natal death (2 weeks). Severe blisters and erosions, hair loss, growth retardation.
	ColXVIII	Viable. Abnormal blood vessels in eyes, neuromuscular junction defects, synapse disorganization.
	ColXIX	Post-natal death (3 weeks). Malnourished, smooth muscle functional defects, inhibited smooth muscle transdifferentiation in the esophagus.
Elastin	-	Post-natal death (4 days). Obstructed, stiff, and tortuous arteries.
Fibrillin	Fbn1	Post-natal death (3 weeks). Cardiovascular defects.
	Fbn2	Viable. Syndactyly, defective mesenchymal differentiation, notochord morphogenesis.
Fibulin	Fibulin-1	Perinatal lethal. Hemorrhages, blood loss, vascular, lung, and kidney defects.
	Fibulin-3	Viable. Reduced reproductivity, early-onset aging.
	Fibulin-4	Perinatal lethal. Severe elastinopathy in lungs and vasculature.
	Fibulin-5	Viable. Loose skin, excessive abdominal folds, expanded lungs, vascular anomalies.
Vitronectin	-	Viable and normal.
Tenascin	Tn-C	Viable. CNS defects, abnormal locomotive behavior, neural crest migration defects.
	Tn-R	Viable. Defects in perineural nets and optic nerve.
	Tn-X	Viable and normal.
Perlecan	-	40% embryonic lethal (E10.5), 60% death after birth. Defective cephalic development, broad and bowed long bones, narrow thorax, craniofacial abnormalities, severe cartilage defects.
Versican	-	Embryonic lethal (E10.5). Heart defects, neural crest migration defects.
Aggrecan	-	Viable. Cleft palate, short limbs, tail, and snout; cartilage defects.
Neurocan	-	Viable. Mild defects in synaptic plasticity.
Brevican	-	Viable. Mild defects in long-term potentiation maintenance.

Table 1- ECM functions in development (Rozario & DeSimone , 2010).

The examples above of ECM genes do not explain a specific role in hematoendothelial development but highlights a general role in early embryo development. The most familiar developmental function attributed to ECM is cell migration (Rozario & DeSimone , 2010).

The dynamic composition and spatial organisation of the ECM provides cues that guide HEP cell migration, adhesion, and localisation within the embryo. Moreover, the ECM's involvement in regulating cellular processes such as proliferation, differentiation, and morphogenesis shapes the developmental trajectories of HEPs as they differentiate into hematopoietic and endothelial lineages. Specific ECM genes have not been explicitly linked to HEP cell development, however, their general functions in promoting cell migration, adhesion, and morphogenesis underline the fundamental importance of ECM-cell interactions in arranging the journey of HEP cell lineages.

This investigation will focus on one ECM gene, Podocalyxin (PODXL) and its potential function in hematopoietic and endothelial differentiation.

3.5 Structure and expression of PODXL

Podocalyxin (PODXL; also known as podocalyxin-like protein 1) is a type I transmembrane sialomucin and a member of the CD34 family. PODXL is a cell surface glycoprotein with a large, highly glycosylated extracellular domain, a single-pass transmembrane region, and a conserved cytoplasmic tail containing a C-terminal PDZ-binding motif (DTHL) (Le Tran, Wang and Nie, 2021) (Fig. 16). Because its extracellular domain interfaces with the extracellular environment and can modulate cell–matrix interactions, it can be described as ECM-associated, but it should not be referred to as an ECM component.

PODXL is expressed in multiple normal cell types including kidney podocytes, vascular endothelium, platelets, haematopoietic progenitors, embryonic stem cells, and subsets of neurons (Le Tran, Wang and Nie, 2021). It is also upregulated in several malignancies, and elevated expression is frequently associated with aggressive phenotypes (Le Tran, Wang and Nie, 2021).

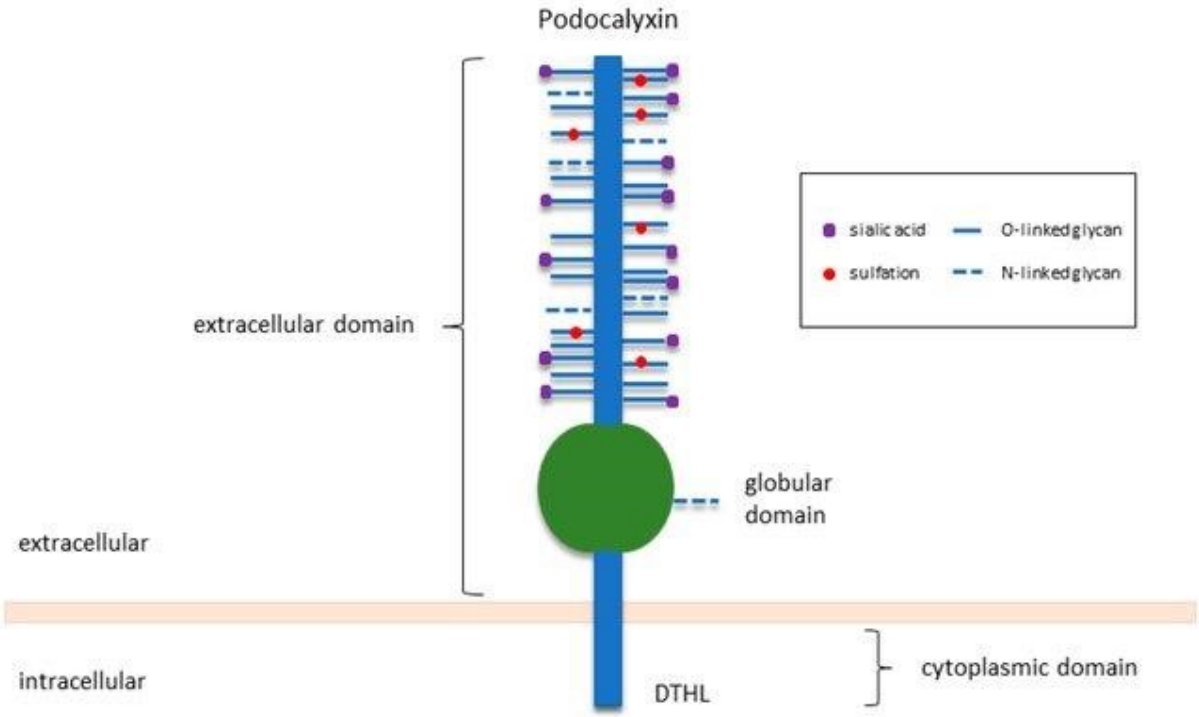

Figure 16- Figure 16. Domain organisation of PODXL, highlighting the highly glycosylated extracellular domain and PDZ-binding cytoplasmic tail. (Le Tran, Wang and Nie 2021).

PODXL expression is regulated at multiple levels. Transcriptionally, it is positively regulated by WT1 and SP1, both of which are involved in growth control, differentiation, and angiogenesis (Le Tran, Wang and Nie, 2021). In contrast, regulators such as p53, PINCH1, and KLF4 act as repressors in specific cellular contexts. PINCH1, an adaptor protein involved in integrin-mediated adhesion and epithelial–mesenchymal transition (EMT), suppresses WT1-driven PODXL expression in podocytes. KLF4 represses PODXL transcription by binding directly to its 5′ untranslated region in gastric cancer cells.

Epigenetic mechanisms also modulate PODXL expression. CpG methylation of the PODXL promoter markedly reduces transcriptional activity in vitro (Butta et al., 2006), whereas promoter hypomethylation has been associated with aggressive behaviour in oral squamous cell carcinoma.

3.5.1 cellular functions of PODXL

Podocalyxin (PODXL), also referred to as podocalyxin-like protein 1, is a type I transmembrane glycoprotein belonging to the CD34 family of sialomucins (Doyonnas et al., 2005; Le Tran, Wang and Nie, 2021). Importantly, PODXL is not an extracellular matrix (ECM) protein. Instead, it is a cell surface protein with a large extracellular domain that interfaces with the extracellular environment and modulates cell–cell and cell–matrix interactions (Doyonnas et al., 2005; Le Tran, Wang and Nie, 2021). Because of this positioning, PODXL is best described as ECM-associated, rather than a structural ECM component.

Structurally, PODXL consists of an extensively O-glycosylated and sialylated extracellular domain, a single-pass transmembrane domain, and a conserved cytoplasmic tail containing a

C-terminal PDZ-binding motif (DTHL) (Larrucea et al., 2008; Le Tran, Wang and Nie, 2021). The dense negative charge of the extracellular domain, conferred by sialylation and sulphation, is a key feature of PODXL and underlies many of its biological functions (Doyonnas et al., 2005).

PODXL is known to regulate cell adhesion, migration, and cell polarity depending on cellular context (Larrucea et al., 2008; Fernández et al., 2013). Its extracellular domain can reduce non-specific cell–cell adhesion through electrostatic repulsion, while its cytoplasmic tail links PODXL to the actin cytoskeleton and intracellular signalling machinery (Larrucea et al., 2008; Takeda et al., 2003). As a result, PODXL does not function as a classical adhesion molecule but rather modulates adhesive behaviour depending on cellular context (Fernández et al., 2013).

Experimental studies have demonstrated that PODXL-mediated effects on adhesion and migration require activation of the small Rho GTPases Rac1 and Cdc42 (Fernández et al., 2013). Silencing Rac1 almost completely abolishes PODXL-dependent promoter adhesion, highlighting the importance of cytoskeletal coupling in PODXL function (Fernández et al., 2013). Therefore, these findings show that PODXL can appear anti-adhesive in some contexts while promoting regulated adhesion and migration in others (Larrucea et al., 2008; Fernández et al., 2013).

PODXL is expressed in a variety of normal cell types, including kidney podocytes, vascular endothelial cells, platelets, haematopoietic stem and progenitor cells (HSPCs), embryonic stem cells, and subsets of neurons (Doyonnas et al., 2005; Le Tran, Wang and Nie, 2021). This broad expression pattern supports a general role for PODXL in regulating cell surface organisation and cellular plasticity across multiple tissues (Le Tran, Wang and Nie, 2021).

3.5.2 PODXL in vasculogenesis and haematopoiesis

During embryonic development, PODXL is prominently expressed in endothelial cells and early haematopoietic progenitors, consistent with the shared haematoendothelial origin of these lineages (Doyonnas et al., 2005; Zhang et al., 2014b). In murine embryos, PODXL expression is detected between embryonic days E10 and E12 in the yolk sac and peripheral blood and decreases as haematopoietic cells undergo terminal differentiation (Doyonnas et al., 2005; Le Tran, Wang and Nie, 2021). Similar expression patterns have been reported in chick embryos, where PODXL is present in haemangioblasts and early haematopoietic stem cells but is largely absent from mature blood cell populations (Zhang et al., 2014b).

This temporal regulation suggests that PODXL functions primarily during early progenitor stages, potentially facilitating migration, vascular association, and niche colonisation, rather than later lineage-restricted differentiation (Zhang et al., 2014b; Amo et al., 2022). In the vasculature, PODXL contributes to endothelial surface organisation and vascular lumen maintenance and limits inappropriate cell–cell adhesion (Amo et al., 2022). PODXL also facilitates transmigration of haematopoietic cells across the endothelium, highlighting its role at the interface between blood and vascular systems (Amo et al., 2022).

In specialised blood vessels called high endothelial venules, PODXL can bind to L-selectin and helps immune cells slow down and attach to the vessel wall during blood flow, supporting immune cell recruitment (Sassetti et al., 1998). Mice that lack PODXL do not show major blood vessel defects, but some develop oedema, suggesting that PODXL contributes to maintaining the endothelial barrier, with other related proteins partly compensating for its loss (Doyonnas et al., 2005; Amo et al., 2022).

At the molecular level, PODXL affects cell polarity and movement by interacting with ezrin and other ezrin–radixin–moesin (ERM) proteins, which connect membrane proteins to the actin cytoskeleton (Takeda et al., 2003; Amo et al., 2022). Through these interactions, PODXL activates signalling molecules such as Rac1 and Cdc42 that help reorganise the cytoskeleton during cell movement and adhesion (Takeda et al., 2003; Fernández et al., 2013). Overall, this allows PODXL to influence how cells change shape and move in a controlled way during development.

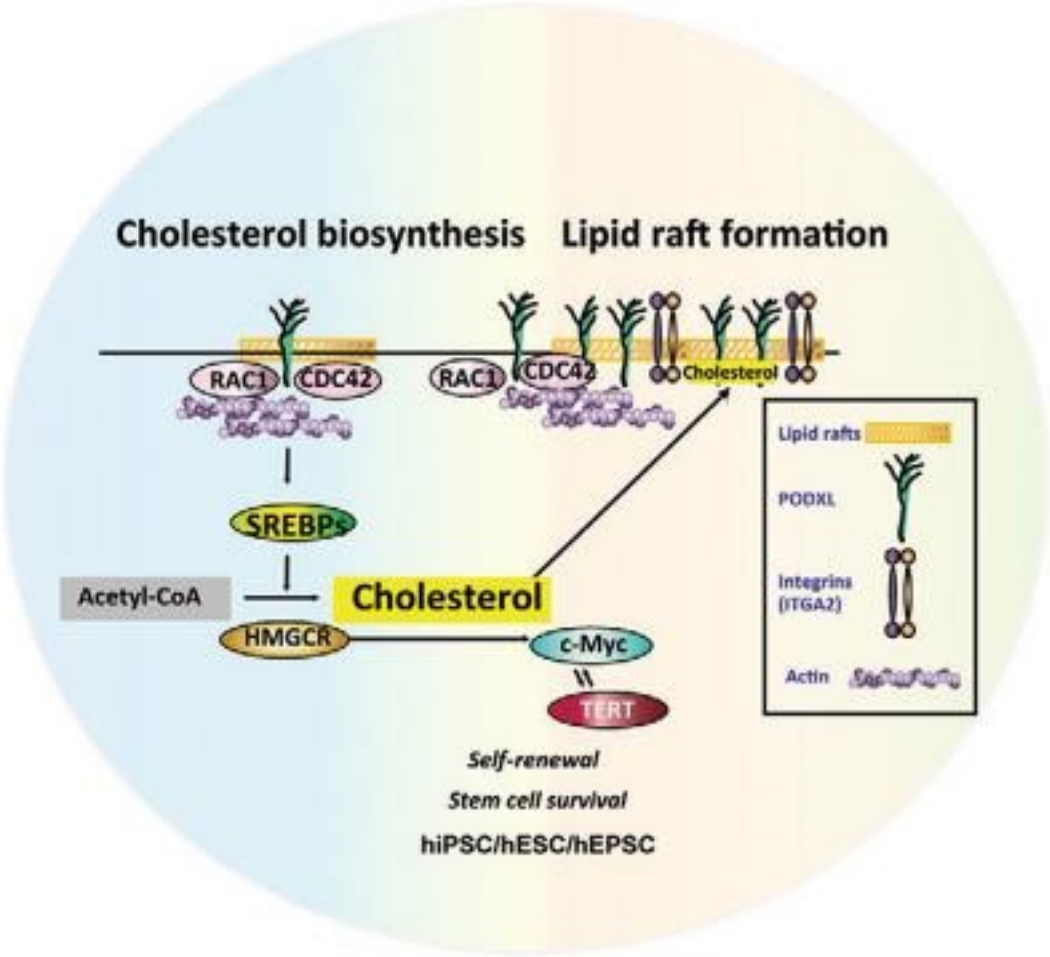

Figure 17- PODXL regulates stem cell pluripotency and survival through the cholesterol biosynthesis pathway and lipid raft formation (Chen, W J 2022)

3.6 Conclusions

Therefore, PODXL is a multifunctional cell surface protein whose effects on adhesion, migration, and polarity are highly context dependent. PODXL enhances adhesion/migration of CHO cells and activates of Rac1/Cdc42 GTPases. PODXL contributes to HSPC trafficking, possibly facilitating nice colonization, while also supporting vascular lumen maintenance.

Though not essential for hematopoiesis or vascular development, its expression is indicative of active cellular remodelling.
In cancer, increased PODXL expression correlates with tumour aggressiveness, invasion, and poor prognosis, particularly in hematologic malignancies. It supports oncogenic signaling, metabolic adaptation, and immune invasion, making it a potential therapeutic target or biomarker in various cancers, including leukemias and B-cell lymphomas.

3.7 The ECM during cardiovascular development.

During heart development, the extracellular matrix (ECM) plays an important role in controlling when and where different cellular processes occur. Early in development, the cardiac ECM is highly hydrated and rich in signalling molecules, but as development progresses it becomes more structured, forming a collagen-rich network that provides mechanical support (Silva, 2020). The composition and organisation of the ECM vary between tissues, and disruption of ECM components can contribute to diseases such as cardiomyopathy, fibrosis, and cancer.

The heart has very limited regenerative capacity in mammals, which contributes to the high prevalence of cardiovascular disease (Sakaguchi, Nishiyama and Kimura, 2020). Proteomic studies show that the cardiac ECM is mainly composed of a small number of proteins, including collagens (types I, III, and IV), fibronectin, laminin, proteoglycans, glycosaminoglycans, and elastin (Silva et al., 2021). Collagen type I makes up most of the fibrillar matrix, with collagen type III also contributing significantly, while collagen IV and fibronectin anchor this matrix to myocardial cell basement membranes (Silva et al., 2021).

The vertebrate heart initially forms as a simple linear tube consisting of an inner endothelial layer and an outer muscular layer (Glickman and Yelon, 2002; Stainier, 2001). Myocardial precursor cells first differentiate in the lateral regions of the embryo and then migrate toward the midline, where they fuse with endocardial cells to form the heart tube. When this migration is disrupted, a condition known as cardia bifida occurs, in which two separate hearts develop. Studies in zebrafish have shown that mutations affecting fibronectin, such as the *natter* mutant, lead to cardia bifida due to loss of fibronectin during myocardial precursor migration (Trinh and Stainier, 2004). Similarly, fibronectin knockdown using antisense morpholinos also causes cardia bifida, highlighting the importance of fibronectin in heart formation (Matsui et al., 2007).

Fibronectin is deposited in two main locations during myocardial precursor migration: on the basal surface of the lateral plate mesoderm, where myocardial precursors reside, and at the embryonic midline between the endoderm and endocardial precursors (Trinh and Stainier, 2004). Experimental injection of fibronectin into the midline region can partially rescue cardia bifida in fibronectin-deficient embryos, suggesting that midline fibronectin helps guide myocardial precursor cells toward the centre of the embryo (Matsui et al., 2007). However, studies of mutants lacking midline fibronectin but retaining fibronectin around the lateral plate mesoderm show delayed, rather than completely blocked, migration, indicating that fibronectin mainly affects the timing and coordination of migration rather than being absolutely required (Trinh and Stainier, 2004).

Additional ECM components also contribute to myocardial migration. Loss of the transcription factor *Mtx1*, which regulates fibronectin expression, results in cardia bifida and reduced laminin

deposition, suggesting that multiple ECM proteins act together to support myocardial precursor migration (Sakaguchi et al., 2006; Rozario and DeSimone, 2010).

Fibronectin also plays a role in organising the polarity of myocardial precursor cells. These cells migrate as a coordinated group and form polarised epithelia during heart tube formation. In *natter* mutants, polarity markers such as aPKC and ZO-1 are mislocalised, and myocardial precursors fail to organise properly, demonstrating that cell–ECM interactions are essential for establishing polarity and effective migration (Trinh and Stainier, 2004).

In parallel with heart development, haematopoietic stem and progenitor cells (HSPCs) emerge from the haemogenic endothelium of the dorsal aorta. This process is regulated by signalling pathways such as VEGF, Notch, BMP, and TGF-β (Butko et al., 2015). In zebrafish, HSPCs bud from the dorsal aorta between 32 and 60 hours post-fertilisation and migrate to the caudal haematopoietic tissue, where they expand. This migration requires ECM remodelling, including local ECM degradation mediated by macrophages and vascular cells, allowing HSPCs to move into circulation.

Overall, these studies show that the ECM plays a central role in cardiovascular development by regulating cell migration, polarity, and tissue organisation, while also influencing haematopoietic stem cell emergence and movement.

4. Preliminary data

Preliminary data shows Mok lab conducted single-cell RNA-sequencing (scRNA-seq) on cells isolated using fluorescence-activated cell sorting (FACS) and labelled with a fluorescent enhancer specific to hematoendothelial progenitors (HEPs) (Figure 6). Through bioinformatic analysis, they observed an enrichment of extracellular matrix (ECM)-associated genes during the early stages of HEP commitment, particularly during gastrulation. Previous findings from their research demonstrated the exclusive expression of ECM genes, including Hapln1, Vcan, Fbln2, and Podxl, in the lateral plate mesoderm, where HEPs are located. This preliminary investigation suggests a potential link between ECM and HEP development, indicating that ECM-associated genes may play a crucial role in regulating HEP commitment and differentiation (Mok *et al*. 2021). The figure below (fig.18) shows Tal1-enhancer reporter construct injected into embryos, which allowed for visualisation of fluorescent cells emerging from dorsal aorta, cardiac crescent and endothelial cells; differentiated endothelial and hematopoietic lineages.

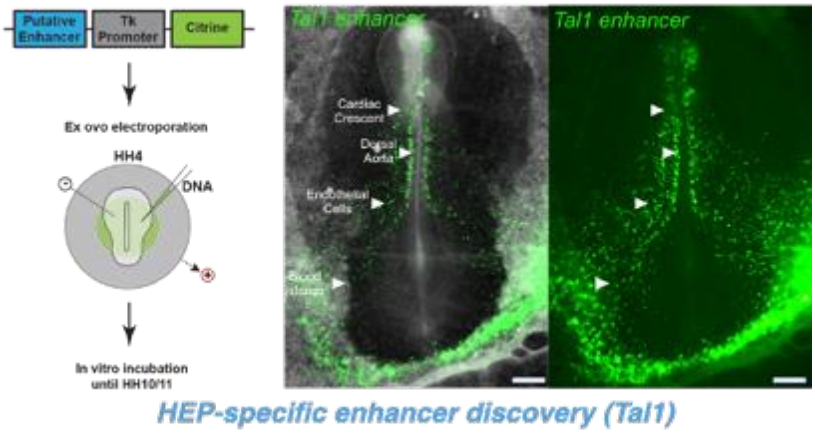

Figure 18- Putative Tal1-enhancer reporter construct created and injected into embryos allows visualisation of HEPs and differentiated endothelial and hematopoietic lineages.

For this investigation, we will use Tal1 as a marker for haematopoiesis when elucidating the role of ECM genes. Tal1 (SCL/ TAL1, T-cell acute leukemia protein) is a transcription factor involved in the process of haematopoiesis and leukemogenesis (Vagapova et al., 2018). It comprises of a helix-loop-helix domain which binds to DNA through its regulatory regions, interacting with the E-box sequence (CANNTG, where N is any nucleotide), and GATA, Ets, and Runx factor binding sites

The Tal1 gene locus is located on human chromosome 1. It contains 6 exons, including the coding exons 4-6. Studies have shown that it is essential for normal embryogenesis and its expression starts on the 7th day after fertilisation. TAL1 expression has been found in the blood islet cells of the yolk sac, endothelial cells, and angioblasts, and then in the liver and spleen of a fetus, the major hematopoietic organs in embryogenesis (Vagapova et al., 2018).
TAL1 is essential for producing hemogenic endothelial cells from mesoderm and later regulates the differentiation of hematopoietic progenitors into red blood cells, megakaryocytes, and platelets. It is also expressed in blood vessel-forming cells during embryogenesis. TAL1 deficiency leads to impaired haematopoiesis and early embryonic death. In murine models,

embryonic stem cells (ESCs) lacking TAL1 cannot differentiate into hematopoietic cells, while ectopic TAL1 expression induces their formation. ESCs without TAL1 show poor differentiation into erythroid progenitors and fail to form lymphoid and myeloid progenitor colonies in vitro (Porcher, Chagraoui and Kristiansen, 2017)

Whole mount in situ hybridisation (Figure 19 A,B,C) reveals ECM genes, PODXL, Versican and FBln2 expressed in lateral plate mesoderm, somite region and in heart forming region. PODXL will be further characterised through functional experiments to elucidate its role in hematoendothelial progenitor development.

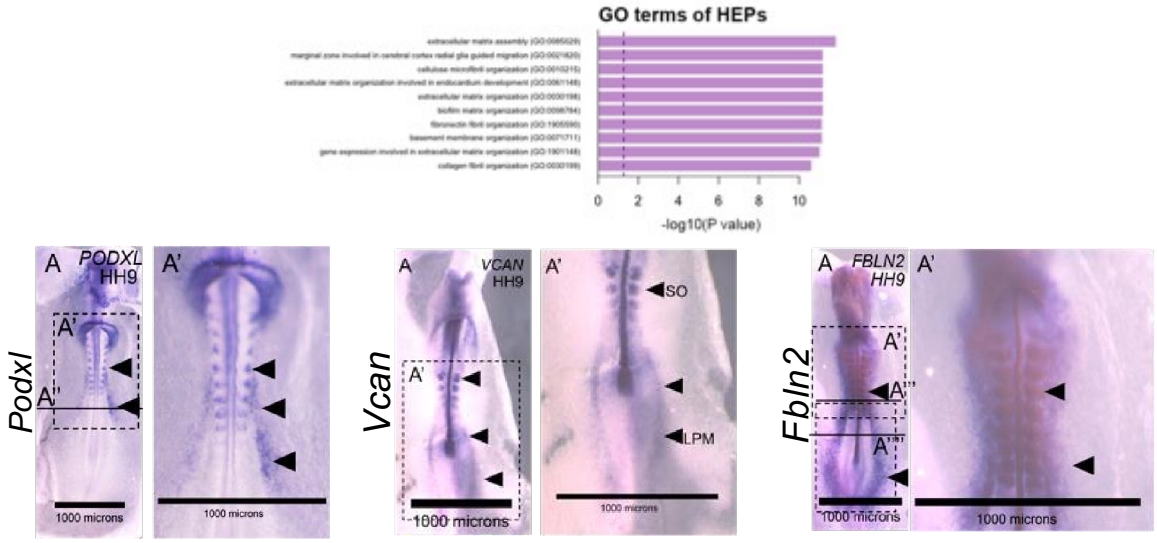

Figure 19- Through bioinformatic analyses, Gene ontology analyses from single RNA seq and subsequent in situ hybridization by Mok lab, have shown enrichment of ECM associated genes in the early stages of HEP commitment during gastrulation and the exclusive expression of ECM genes in the LPM. This suggests that it may play a role during the early development of Hematoendothelial progenitor development (Pouncey, L, Mok, GF 2021).

The preliminary data from Mok lab's scRNA-seq analysis highlighted a significant enrichment of ECM-related genes in the lateral plate mesoderm during early HEP commitment. These findings suggest that ECM components may be involved in the regulation of hematoendothelial progenitor development. Among the numerous ECM genes identified, PODXL**,** Fbln2**,** Vcan**,** and Hapln1 emerged as particularly interesting candidates due to their distinct expression patterns and potential roles in cell adhesion, signalling, and tissue structure.

5. Aims and Hypothesis

Hypothesis

We hypothesise that Podocalyxin (PODXL) plays an important role in the development and early commitment of haemato-endothelial progenitor (HEP) cells. PODXL is known to regulate cell adhesion, polarity, and migration, which are key processes during mesodermal cell movements and HEP formation.

Preliminary expression data show that ECM-associated genes are enriched in the lateral plate mesoderm, a region where HEPs arise. Based on this, we propose that disrupting Podxl expression will affect normal haemato-endothelial development. Changes in the expression of Tal1, a key regulator of haemato-endothelial specification, will be used to assess the impact of Podxl disruption on HEP development.

Aims

Aim 1- To characterise the spatial and temporal expression of Podxl during HEP development
This aim will determine where and when Podxl is expressed during early chick development. In situ detection of Podxl mRNA will be performed using Hybridisation Chain Reaction (HCR) on embryos collected between Hamburger and Hamilton stages HH7–HH12. This will define the relationship between Podxl expression, the lateral plate mesoderm, and emerging haemato-endothelial progenitors.

Aim 2- To determine the functional role of Podxl in HEP development
To assess the functional importance of Podxl, splice-blocking morpholinos will be used to disrupt normal Podxl mRNA splicing. These morpholinos bind exon–intron junctions, preventing correct splicing and leading to reduced or non-functional PODXL protein. The effects of Podxl knockdown on haemato-endothelial development will be assessed using molecular markers, including Tal1, as a readout of HEP specification and early haematopoiesis.

Aim 3- To identify cis-regulatory elements controlling Podxl expression during development
This aim will investigate how Podxl expression is regulated at the transcriptional level. Candidate enhancer regions will be selected using ATAC-seq data and cloned upstream of a GFP reporter. These constructs will be tested in chick embryos to determine whether they drive tissue-specific Podxl expression. This approach will help define the regulatory logic that controls Podxl expression during haemato-endothelial development.

6. Methods and materials

6.1 Incubation of Chicken eggs

Fertilised chicken eggs were incubated at 37°C, in conditions of approximately 70% humidity (*timing can vary according to season and egg quality). Chicken eggs were supplied by Henry Stewart and Co. before incubation, each egg is placed with its long axis orientated horizontally. All embryos were staged according to Hamburger and Hamilton (HH). Embryos would only develop to HH14. HH3-4 embryos would be needed (18-24 hours incubation, depending on incubator) for injection and electroporation.

6.2 Albumin- agar plates

A mixture of Agar Agar in RB buffer (6 g/L) was heated in a microwave in 30-second intervals until the Agar Agar was completely dissolved. The resulting agar solution was then transferred into 50ml Falcon tubes and allowed to cool in a water bath maintained at 37°C. 500 µL of penicillin-streptomycin (PenStrep) was added per 50mL of agar solution (once agar solution reached desired temperature). The mixture was inverted several times to ensure uniform antibiotic distribution. Finally, the agar solution was poured into sterile petri dishes and allowed to solidify, forming the Albumin-agar plates.

6.3 Embryo culture

Following incubation, the eggs were cooled for 1 hour to return them to room temperature prior to experimentation. Embryos were harvested using the filter paper ring method as part of the easy culture technique. To begin, the top of the egg was gently cracked with giant forceps, and the shell was carefully removed to expose the yolk. The thick albumin was then removed by using forceps to pull it upwards and away from the shell, thereby preventing the yolk from being dragged along the edges of the eggshell, which could potentially cause damage. Residual thick albumin was carefully removed with tissue paper once the embryo was visible. A Whatman 3MM CHR filter paper ring was placed over the embryo, and fine scissors were used to cut the vitelline membrane around the filter paper. The filter paper, with the attached embryo, was gently pulled away from the yolk in an oblique direction. Any residual yolk was washed off with Ringer's buffer. The filter paper, with the embryo oriented ventral side up, was then placed in an easy-culture dish (35 mm Falcon) containing the Albumin-agar solution.

6.4 Hybridisation Chain Reaction

Probe Design: Probes for the Hybridization Chain Reaction (HCR) were devised to target specific mRNA sequences, PODXL and Tal1, in HH10 chick embryos. The probe sequences were designed by Mok lab to ensure precise binding and minimal off-target interactions.
Preparation of fixed whole-mount chicken embryos: HH10 chick embryos were gathered and fixed to maintain mRNA integrity and tissue structure. This was done by allowing fertilised eggs to develop for 48 hours in the incubator to reach stage HH8-11. Embryos were cultured using the filter paper technique and fixed in 4% PFA for one hour. Sections of the tissue were then prepared for dissection. Embryos were first transferred to a dish of 1x PBST before being dissected off the filter paper. The fixed vitelline membrane (opaque) was removed and cut around the area pellucida, without leaving excess of extra embryonic tissue. Dissected embryos were then washed in 2mL 1x PBST in a glass tube- nutated for 5 minutes with the tube positioned horizontally on ice. Embryos were washed two times and then dehydrated with

2 x5 min washed of 2 mL 100% methanol (MeOH) on ice. Finally, embryos were stored at -20 overnight before continuing with protocol.

Embryos would then be rehydrated with a series of graded 2 mL MEOH/ PBST washes for 5 minutes on ice: (a) 75% MeOH/25% PBST, (b) 50% MeOH/ 50% PBST (c) 25% MeOH/ 75% PBST (d) 100% PBST x2. Embryos were then treated with 10 ug/mL proteinase K for two minutes (stage HH8) or 2.5 minutes (stage HH10-11) at room temperature. Following treatment, embryos were postfixed with 2 mL of 4% PFA for 20 minutes at room temperature and then washed 2 x 5 minutes with 2 mL of PBST on ice. Embryos were further washed with 2 mL of 50 % PBST/ 50% 5x SSCT on ice and then 2 mL of 5xSSCT on ice.

Detection stage: Embryos were pre-hybridised in 500 uL of probe hybridisation buffer supplied by Molecular Instruments for 30 minutes at 37 degrees. The probe solution was prepared by adding 2pmol of each probe set (e.g. 2uL of 1uM stock) to 500 uL of probe hybridisation buffer at 37 degrees. Once the pre- hybridisation was removed, the probe solution was added to the embryos and incubated overnight (12-16 hours) at 37 degrees.

Amplification stage: after hybridisation, embryos were washed 4 x 15 minutes with 1 mL probe wash buffer at 37 to remove excess probes. Embryos were then washed 2 x 5 minutes with 5x SSCT at room temperature. Before tissue sections were amplified with the HCR signal, embryos were washed with 500 uL of amplification buffer for 5 minutes at room temperature. 30 pmol of hairpin h1 and 30 pmol of hairpin h2 were prepared by snap cooling 10 uL of 3 uM stock- hairpins were heated at 95°C for 90 seconds and cooled to room temperature in a dark drawer for 30 minutes. Hairpin solution was prepared by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 500 uL of amplification buffer at room temperature. The preamplification buffer was removed, and hairpin solution was added to embryos. Embryos were incubated overnight in the dark at room temperature before removing excess hairpins and washing with 1 mL of 5x SSCT at room temperature: 2x 5 minutes, 2 x 30 minutes (1 uL DAPI + 2mL 5x SSCT), final 1 x 5 minute. Embryos were stored at 4 degrees protected from light before microscopy.

6.5 Oligonucleotide morpholinos

Splice blocking morpholinos to knock down PODXL and Hapln1
PODXL sequence- GTGTATGCTGGGTCACCAAAATACA
Oligonucleotides were filtered, selectively precipitated, resuspended in pure water, and freeze-dried to remove all contaminants. The oligonucleotides were then dissolved in sterile water to a final concentration of 1 mM and volume 20uL. Splice-blocking morpholino oligonucleotides (MOs) were designed to skip exon 3, which consists of 155 bases. This exon skipping induces a frameshift downstream of the targeted sequence, likely introducing a premature termination codon in-frame. As a result, this modification is expected to trigger nonsense-mediated decay, leading to the degradation of the affected transcript.

Morpholinos were labelled with a far-red tag, Lissamine, to facilitate detection. Following injection into embryos and subsequent development, hybridization chain reaction (HCR) was performed using Tal1 probes. This procedure aimed to determine whether PODXL knockdown is associated with altered Tal1 expression.

6.6 Cloning putative enhancers in Ptk-citrine vector

Candidate enhancer regions associated with PODXL were identified using publicly available single-cell RNA-seq and ATAC-seq datasets, which were analysed to locate regions of open chromatin and potential regulatory activity near the PODXL locus.

Chick genomic DNA was previously isolated from HH12-HH14 chick embryos using the Purelink gDNA mini kit. Embryonic tissue (25 mg) was homogenized in 180 ul digestion buffer and 20 ul proteinase K, followed by incubation at 55°C for 1 hour with periodic vortexing.

Purification of gDNA Following lysis, cell debris was removed by centrifugation, and the supernatant was treated with RNaseA. Genomic lysis/binding buffer and ethanol were added to the lysate, and the mixture was transferred to PureLink spin columns for purification. Wash steps were performed using wash buffers 1 and 2, and gDNA was eluted in elution buffer

PCR Amplification- gDNA was used as a template for PCR amplification of putative enhancer sequences using KAPA LR DNA polymerase. PCR cycling conditions included denaturation, annealing, and extension steps: 94°C, 3 minutes x1, 94°C 15 seconds, 55°C, 15 seconds, 68°C, 3 minutes x10, 94 °C, 15 seconds, 63 °C 15 seconds, 68°C, 3 minutes x 25, 72°C, 4 minutes x1. PCR products were visualized on a 1 % gel, and remaining PCR reactions were purified using the Qiagen PCR clean-up kit and then checked on nanodrop to get concentration. BSMBI reaction includes 75ng (Xul) PCR product, 75ng (1.0uL) nanotag vector, T4 DNA ligase buffer (2.0uL), BsmBI (1.0uL), T4 DNA ligase (1.0ul), pure water making up to final volume of 20ul. Reaction followed PCR conditions: 37°C, 2 minutes 25x, 16°C, 5 minutes, 55°C 5 minutes, 80°C, 5 minutes.

Enhancer cloning- PCR products were subjected to enzymatic digestion with BsmBI, followed by ligation into the Ptk vector. The reaction mixture was transformed into DH5alpha chemically competent cells, and transformed colonies were selected on LB agar plates containing ampicillin.

Colony PCR using Xgal/LacZ system- The Ptk vector used for cloning has a reporter gene, lacZ, which encodes for β-galactosidase. X-gal is a colourless substrate that, when cleaved by β-galactosidase, produces a blue product. In a successful cloning event, the enhancer sequence is inserted into the plasmid, disrupting the lacZ gene. As a result, the βgalactosidase enzyme is not functional. When transformed cells with disrupted lacZ gene are plated on X-gal-containing agar plates with a suitable antibiotic (such as carbenicillin), the colonies formed will be white, as they cannot produce the blue colour. If there's a failure in cloning (insert not integrated), the lacZ gene remains intact, and β-galactosidase is produced. Colonies on the plate will appear blue due to the cleavage of X-gal.
Colony PCR (using 1% agarose gel) was performed to screen for positive clones, and selected colonies were subjected to endotoxin-free plasmid preparation using the OmegaBioTek EZNA endofree mini spin prep kit II. Cloned enhancers were sent off to BioSource for sequencing. plasmid DNA was isolated using the Qiagen Maxi Prep kit according to the manufacturer's protocol.

6.7 Ex ovo Electroporation and injecting

Embryos were injected with fluorescent construct/ oligo morpholinos and then electroporated at early gastrulation (HH3-HH4) stage. The electroporation set up involves a negative electrode chamber that resembles a small (~35mm) petri-dish, made of a clear plastic resin. This was filled with Ringer's solution. The solid base is 4mm in depth and features a central blunt cone shaped cavity (~9mm diameter at the top, 4mm at the base) with a platinum foil sheet (0.01 x 5 x 5 mm) situated at the base of this cavity. This chamber has an external copper tab to connect to the electroporator. The positive electrode is a platinum paddle housed in a pen-like structure. After injecting, the electric current was applied, with a set of 5 pulses (5 V, 100 ms ON, 50 ms OFF). The embryo was placed back into the 35mm petri dish (with thin albumin and agar solution) and sealed with thin albumin coating the edge of the lid. Injected embryos would be left to develop in the incubator O/N at 37 degrees.

The needle would be prepared with 2ug/uL of enhancer construct, 2 uL dye and made up to a total volume of 20 uL with TE buffer and transferred to glass needle.

6.8 Confocal microscopy

Whole- mount embryos were prepared and labelled with fluorophore-conjugated probes targeting specific molecules. Nuclei were counterstained with DAPI. Imaging was performed using a laser scanning confocal microscope (Zeiss LSM 980 Airyscan2 with super-resolution capability) using Zen (blue) software. Z-stack imaging enabled three-dimensional visualization. Images were processed and analysed in Fiji (ImageJ)

Chapter 7- Results

7.1 Using Hybridisation chain reaction to visualise the spatial expression of Tal1 in stage HH8 embryo

Tal1 expression was analysed in HH8 chick embryos using Hybridisation Chain Reaction (HCR) to define its spatial distribution during early haematoendothelial development. HH8 embryos were selected as this stage corresponds to early lateral plate mesoderm (LPM) patterning. Confocal imaging revealed Tal1 mRNA expression in discrete regions of the embryo (Fig. 20). Tal1 signal was detected within the lateral plate mesoderm and in areas corresponding to developing blood island regions (Fig. 20B). Tal1 expression was also observed adjacent to the paraxial mesoderm; however, the signal indicated that it is present in the LPM rather than the paraxial mesoderm itself (Fig. 20B, arrow).Higher-magnification imaging confirmed Tal1-positive cells within the LPM, with no detectable expression in the neural tube (Fig. 20C). These observations indicate that at HH8, Tal1 expression is enriched in mesodermal regions associated with early haematoendothelial progenitor development.

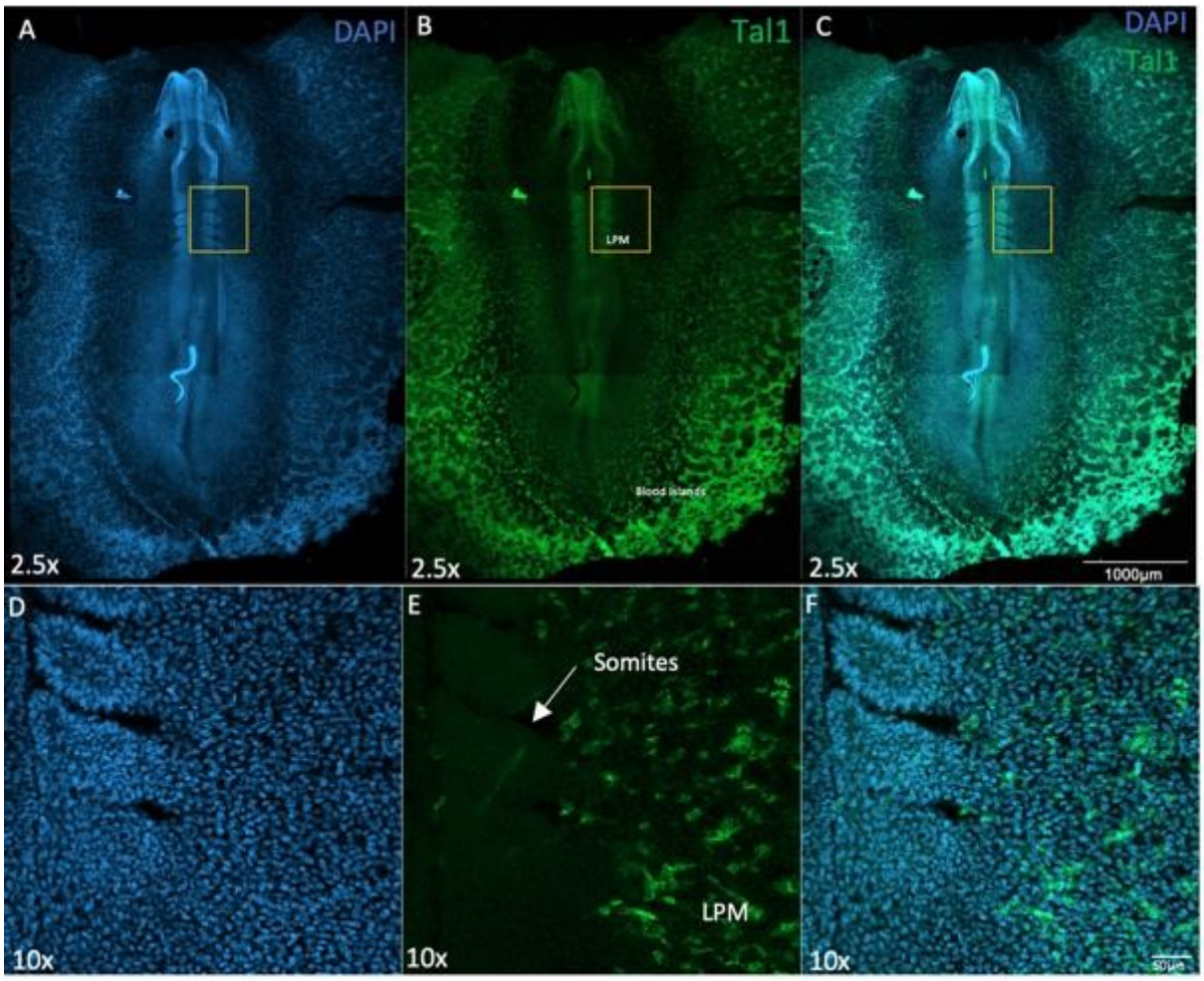

Figure 20- Spatial expression pattern of Tal1 in HH8 chick embryo using Hybridisation chain reaction to visualise Tal1 (green) mRNA.

DAPI (blue) used to counterstain and visualise cell nuclei at 2.5x and 10x of highlighted region (yellow box) (A). HCR staining for Tal1 (green) (B) combined with nuclear counterstaining using DAPI (blue) (C) reveals the distribution of Tal1-expressing cells in the embryo Tal1 expression in blood island regions and near somite regions on the lateral plate mesoderm.

Tal1 mRNA is labelled with a specific fluorophore (488) attached to the HCR probes, allowing for the visualization of Tal1 expression in the embryo. Images were processed on ImageJ (fiji). Scale bar- 50um. LPM- Lateral plate mesoderm.

7.1.1Spatial and temporal expression of Tal1 and PODXL in stage HH10 and stage HH11 embryos

Hybridisation Chain Reaction (HCR) was performed to examine the spatial expression patterns of PODXL and Tal1 in HH10 and HH11 chick embryos. Multiplex HCR was used to visualise both transcripts simultaneously.

In HH10 embryos, PODXL mRNA signal was detected in the heart-forming region, lateral plate mesoderm (LPM), and in regions adjacent to the Somites (Fig. 21A). Tal1 expression was observed in the LPM and endothelial-associated regions (Fig. 25B). Merged images show that PODXL and Tal1 signals are present in overlapping regions within the LPM and cardiac region (Fig. 21C).

At HH11, PODXL expression remained visible in the LPM and heart-forming region (Fig. 21A). Tal1 expression was detected in the LPM and regions associated with developing vascular structures (Fig. 21B). Merged images indicate that both transcripts are present within the LPM, although their expression domains are not completely identical (Fig. 21C).

Higher-magnification imaging showed PODXL signal extending toward paraxial mesodermal regions, whereas Tal1 expression remained enriched within the LPM (Fig. 21).

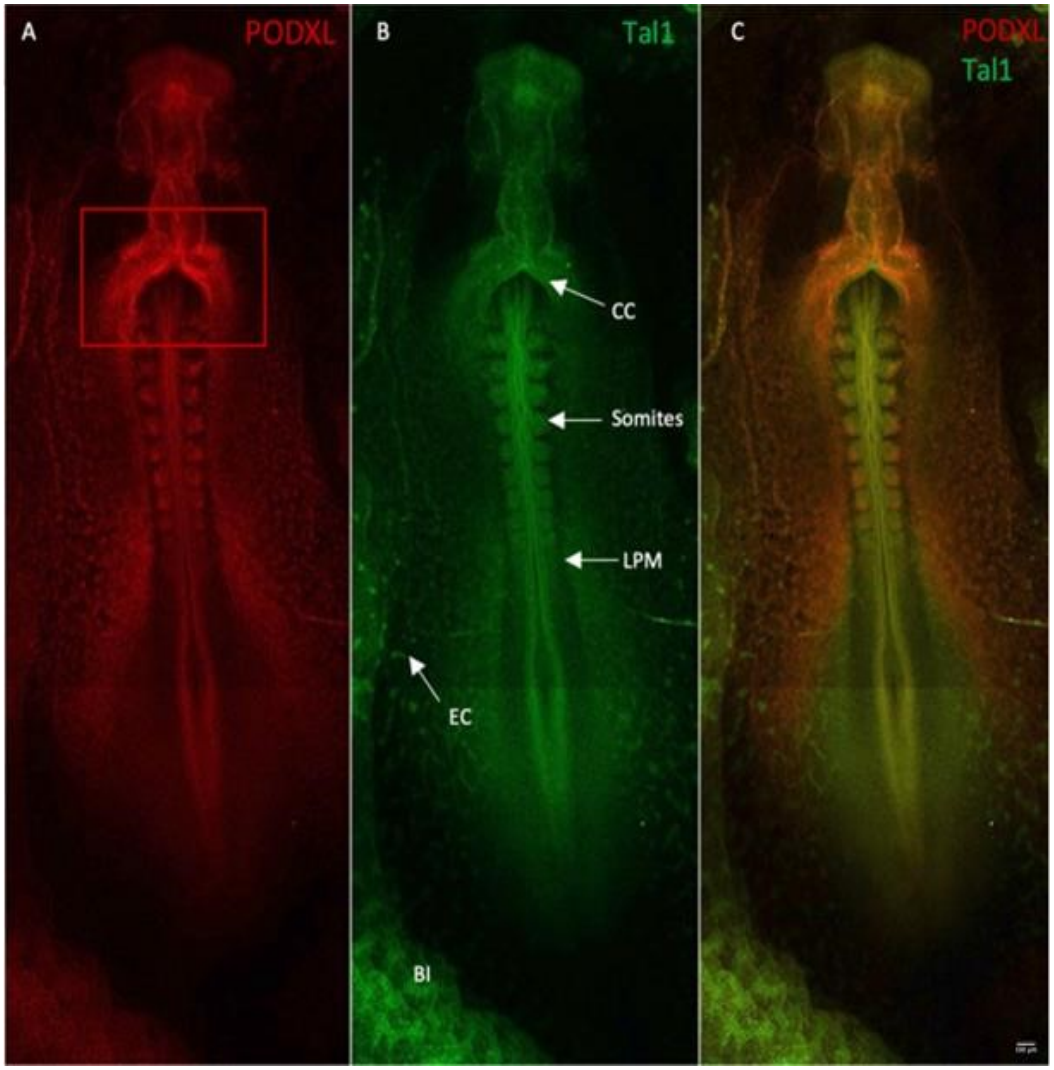

Figure 21- Expression of PODXL and Tal1 mRNA in HH10 embryo.
Confocal images showing expression patterns of PODXL mRNA indicated by red fluorescence in the cardiac regions (A) and Tal1 mRNA indicated by green fluorescence in endothelial cells (B) in stage HH10 chick embryos. Embryos developed to HH10 in incubator and PODXL and TAL1 expression visualised using Hybridisation Chain Reaction with PODXL specific probes (Alexa Fluor 546) and TAL1 (Alexa Fluor 488) Scale Bar = 100um

7.1.2 HCR analysis reveals PODXL and Tal1 expression in the lateral plate mesoderm of HH11 embryos

Embryos were incubated for 54 hours to reach stage HH11. Hybridisation Chain Reaction (HCR) was performed using probe sets targeting PODXL and Tal1 transcripts.
Confocal imaging revealed PODXL expression in the lateral plate mesoderm (LPM) and heart-forming region (HFR) (Fig. 22A). A higher magnification view showed PODXL signal extending along the LPM adjacent to the neural tube (Fig. 22B).
Tal1 expression was detected primarily within the LPM and endothelial-associated regions (Fig. 22C). The merged image shows that PODXL and Tal1 signals are present in overlapping regions within the LPM and cardiac region (Fig. 22D).

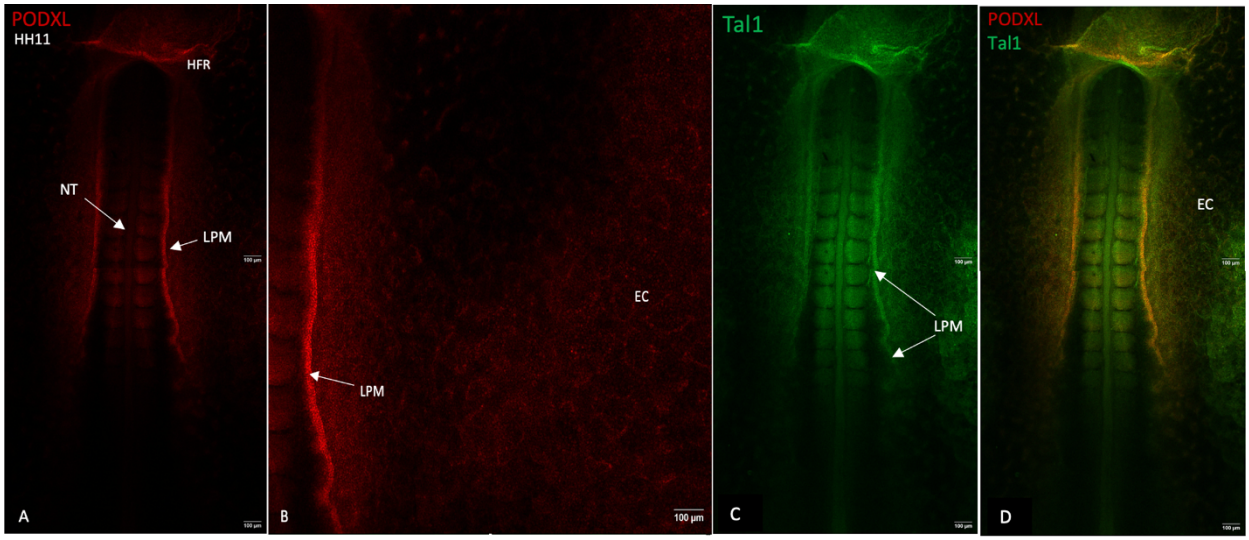

Figure 22. Hybridisation Chain Reaction (HCR) analysis of PODXL and Tal1 mRNA expression in HH11 chick embryos.
(A) PODXL expression detected using Alexa Fluor 546 probes showing signal in the lateral plate mesoderm (LPM) and heart-forming region (HFR).
(B) Higher magnification view highlighting PODXL expression along the LPM.
(C) Tal1 expression detected using Alexa Fluor 488 probes showing signal within the LPM and endothelial-associated regions.
(D) Merged image showing PODXL (red) and Tal1 (green) expression in overlapping regions of the LPM and cardiac region.
Images were captured using confocal microscopy at 2.5× magnification (A, C, D) and 4× magnification (B).
Scale bars = 100 µm.
NT = neural tube; LPM = lateral plate mesoderm; EC = endothelial cells; HFR = heart-forming region.

7.1.3 PODXL expression extends toward paraxial mesoderm while Tal1 remains enriched in the lateral plate mesoderm

Confocal imaging at 10× magnification revealed differences in the spatial distribution of PODXL and Tal1 transcripts in HH11 chick embryos. PODXL signal was detected extending toward the paraxial mesoderm adjacent to the neural tube (Fig. 23A). In contrast, Tal1 expression was primarily observed within the lateral plate mesoderm (LPM) (Fig. 23B). The two transcripts were therefore present in nearby mesodermal regions but did not completely overlap.

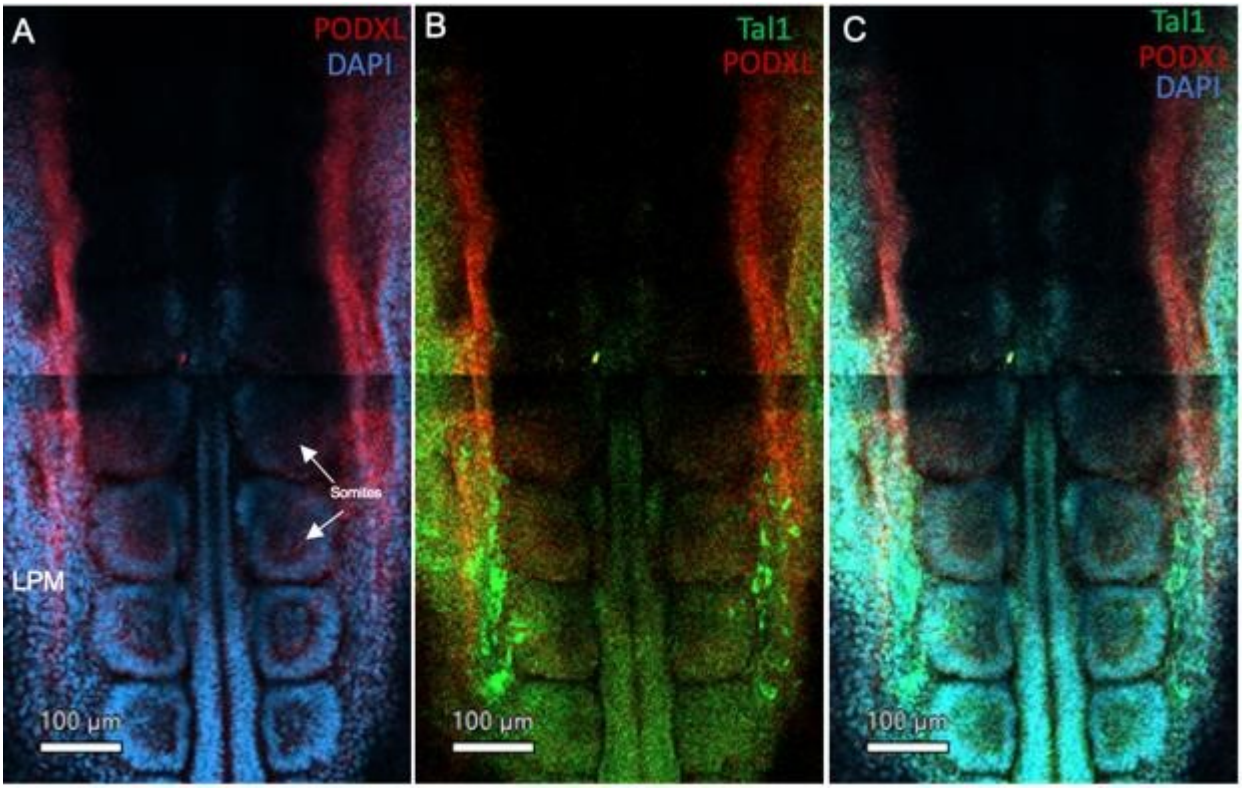

Figure 23- Confocal microscopy images of HH11 chick embryos showing spatial expression patterns of PODXL and Tal1 detected by Hybridisation Chain Reaction (HCR).

(A) PODXL mRNA expression detected using Alexa Fluor 546 probes showing signal extending toward the paraxial mesoderm adjacent to the neural tube.

(B) Tal1 mRNA expression detected using Alexa Fluor 488 probes showing signal within the lateral plate mesoderm (LPM).

Images were acquired at 10× magnification.

Scale bar = 100 µm.

8 PODXL knockdown causes cardiac abnormalities while Tal1 expression remains detectable

Splice-blocking morpholinos targeting PODXL were injected into HH3 chick embryos and embryos were allowed to develop to HH11. Tal1 expression was examined using Hybridisation Chain Reaction (HCR).

Confocal imaging showed Tal1 expression within the lateral plate mesoderm and in regions adjacent to emerging Somites in morpholino-treated embryos (Fig. 24A). Tal1 signal was detected in these mesodermal regions despite PODXL knockdown. DAPI staining was used to visualise nuclei (Fig. 24B), and merged images confirmed the localisation of Tal1-positive cells within the mesoderm (Fig. 24C).

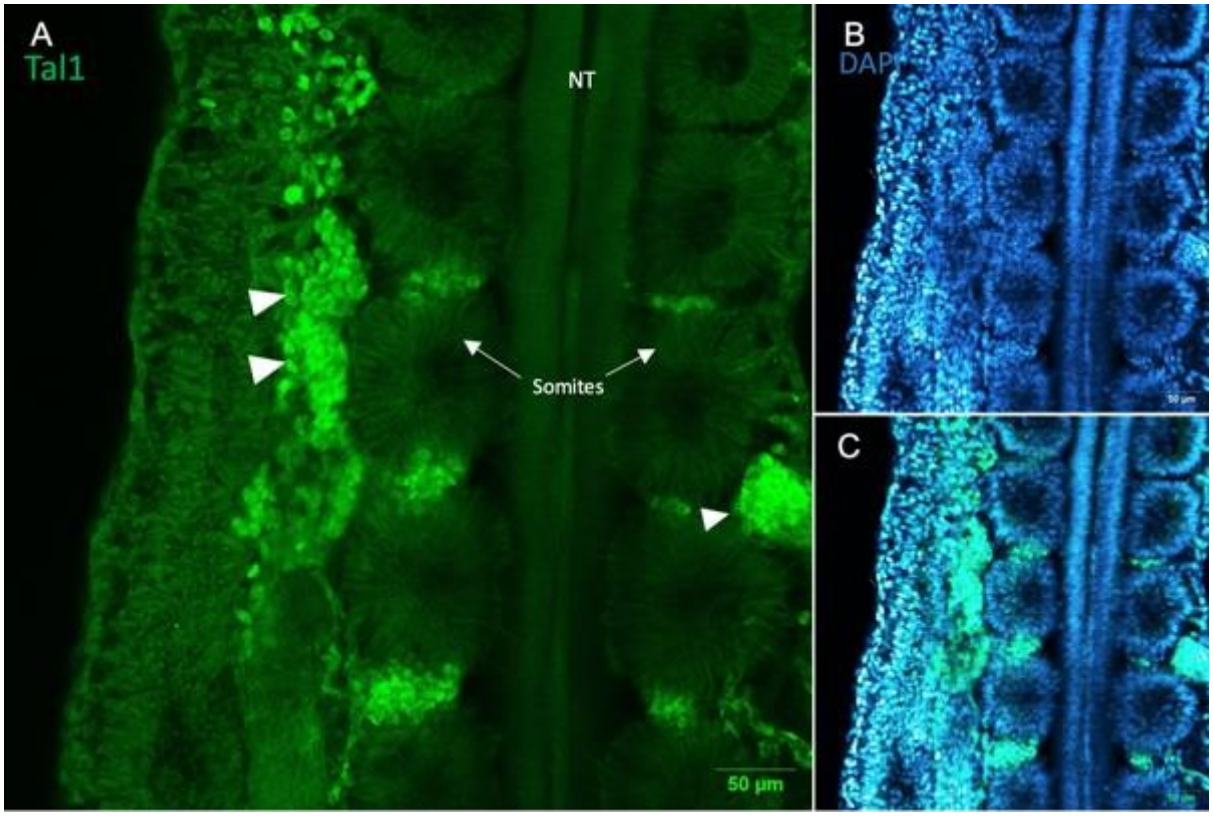

Figure 24 - Confocal images of stage HH11 embryo injected with PODXL morpholino. *Images are of Lateral plate mesoderm and Somites at 10x magnification, with 5 pairs of Somites, neural tube visible from the middle of the embryo. (A) Tal1 expression indicated by green fluorescence coming through the LPM and around third pair of Somites. (B) DAPI staining all cell nuclei. (C) merged image of (A) and (B) with green fluorescence and DAPI.*

10x magnification, scale bar= 50um.

8.1 PODXL knockdown results in cardiac developmental defects

Embryos injected with PODXL splice-blocking morpholinos were examined for morphological abnormalities during development. Fluorescence from the lissamine-tagged morpholino confirmed morpholino uptake in injected embryos (Fig. 25A).
Some embryos displayed abnormal cardiac looping, visible under brightfield imaging (Fig. 25B–C). In addition, a subset of embryos exhibited cardia bifida, where two separate heart structures formed on either side of the embryo (Fig. 25A).
Fluorescence imaging using the RFP filter confirmed the presence of lissamine-tagged morpholino signal in embryos displaying cardiac defects (Fig. 25B–C).

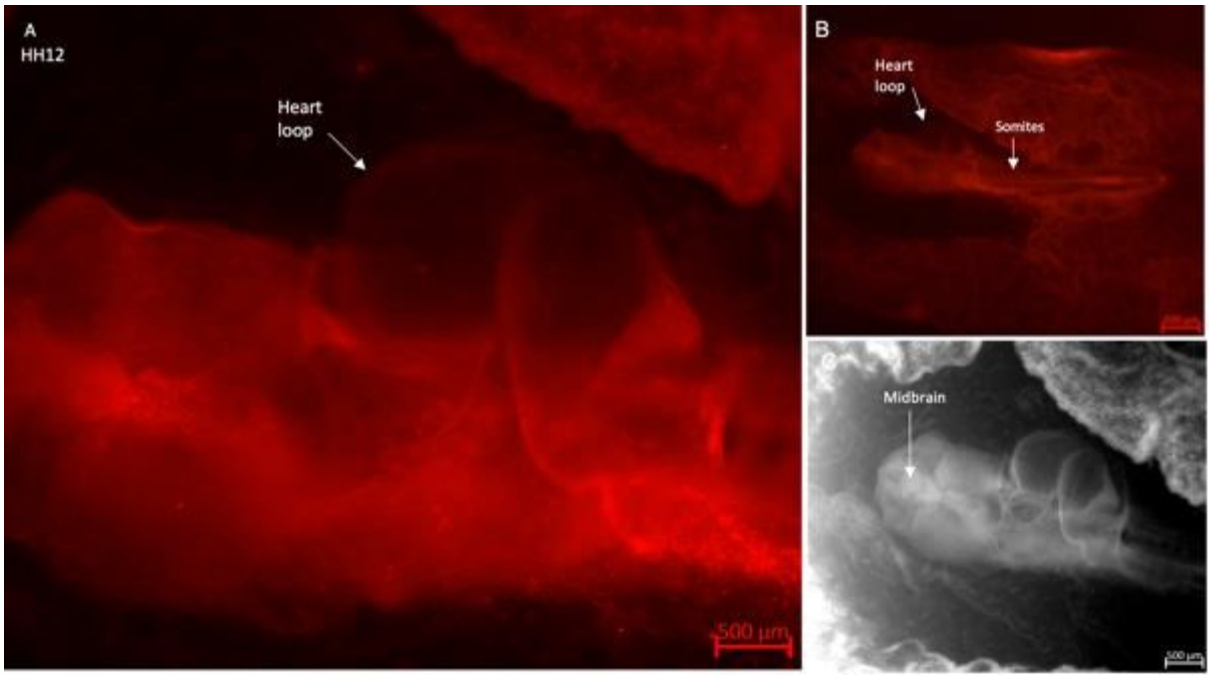

Figure 25- Morpholino uptake and heart developmental defects in chick embryos following PODXL knockdown. Chick embryos were injected with a far-red lissamine-tagged morpholino at the HH3 stage to knock down PODXL and allowed to develop. (A) using a Zeiss stereo discovery.8 stereo microscope with an RFP filter set to detect any florescence at 8x magnification. Abnormal looping of the heart loop is visible. (B) image captured at 2.5x magnification and (C) 4x magnification under brightfield with the abnormal looping of the heart visible. Scale bar= 500um

In addition to abnormal looping, some morpholino-treated embryos displayed cardia bifida, where two separate heart structures were visible on either side of the embryo (Fig. 26A). Fluorescence imaging using the RFP filter confirmed lissamine-labelled morpholino uptake in these embryos (Fig. 26B–C).

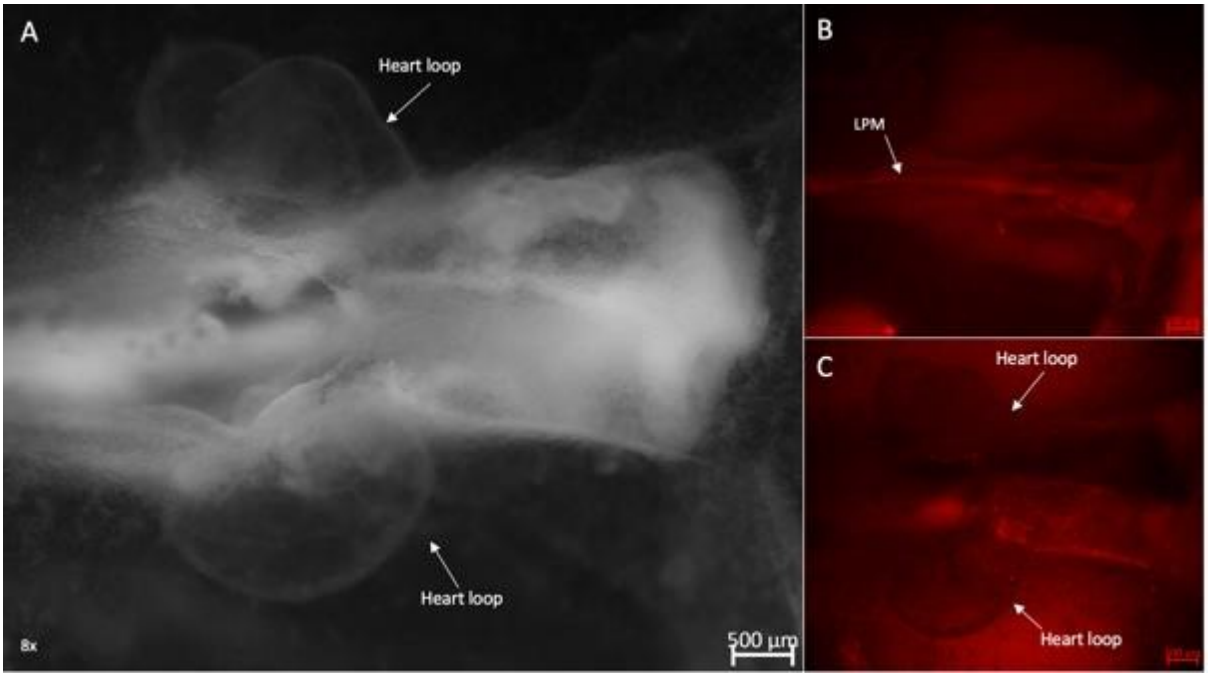

Figure 26- Cardia bifida observed following PODXL knockdown
Images of embryos injected with PODXL splice-blocking morpholino showing cardia bifida. (A) Brightfield image showing two separate heart structures on either side of the embryo. (B–C) RFP filter images confirming lissamine-tagged morpholino uptake in the embryo. Scale bar = 500 µm.

Some embryos injected with PODXL splice-blocking morpholinos displayed an additional cardiac phenotype characterised by abnormal enlargement and exaggerated looping of the heart tube (Fig. 27A). Fluorescence imaging using the RFP filter confirmed lissamine-labelled morpholino uptake in these embryos (Fig. 27B). Brightfield imaging showed the abnormally curved heart tube within the cardiac region (Fig. 27C).

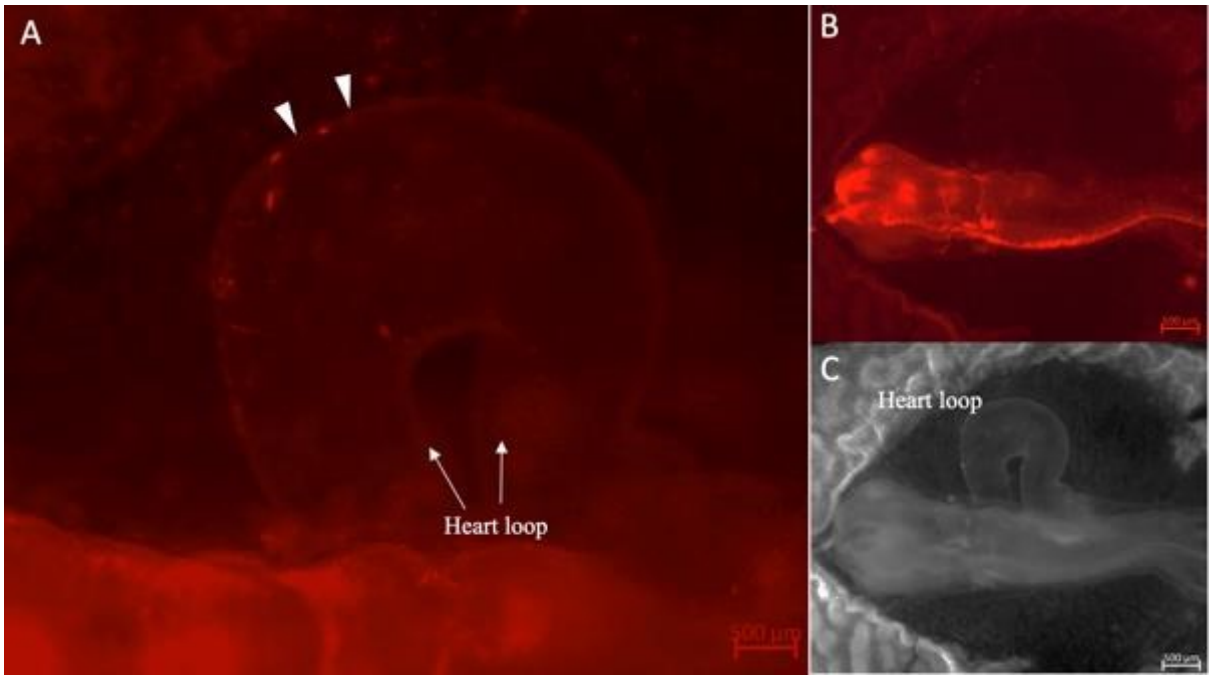

Figure 27- Large, abnormal looping of the heart visible under brightfield and RFP filter.
Embryos were injected with splice-blocking morpholinos at HH3 and then developed to HH11. (A) 8x magnification will show fluorescent cells. (B) 4x magnification showing head and heart tube region with fluorescence, indicating morpholino uptake. (C) 4x magnification of head and heart region of embryo under brightfield. Scale bar- 500um.

8.2 Percentage of Embryos with Abnormal, cardia bifida and Normal Development After PODXL

Embryos were scored for cardiac morphology following PODXL morpholino injection. Among 13 analyzed embryos, 38.4% showed normal heart morphology, 46% displayed abnormal heart looping, and 15.3% exhibited cardia bifida (Fig. 34).

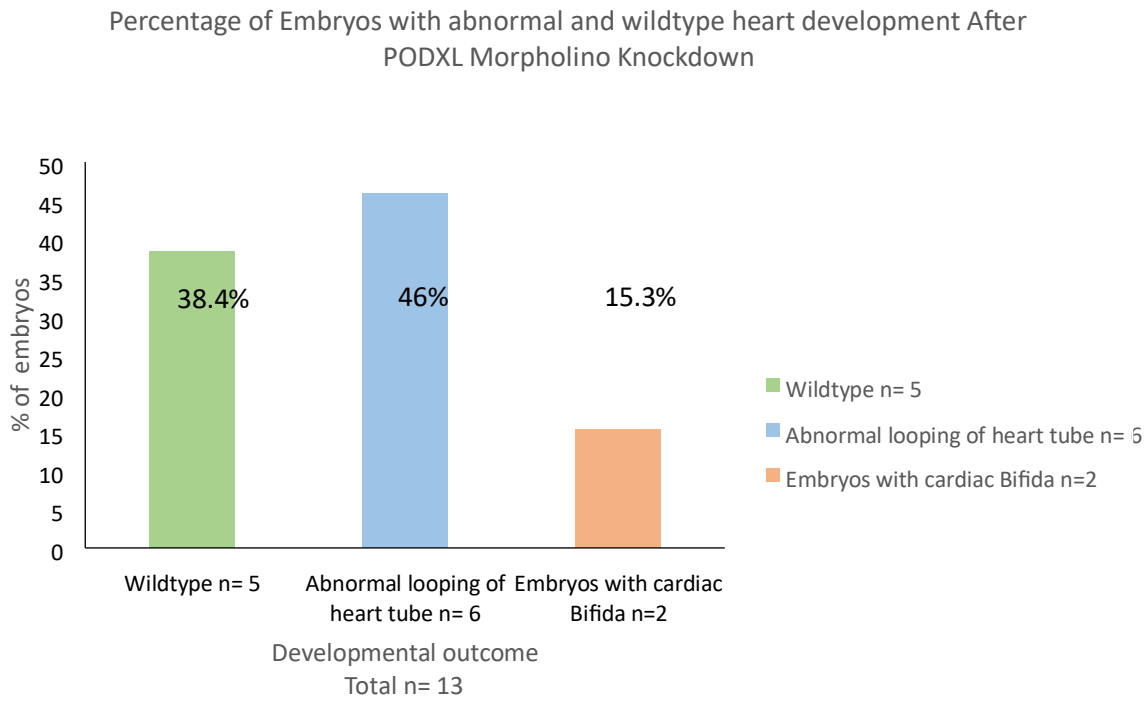

Figure 28- Bar graph showing the percentage of embryos displaying normal, abnormal heart development, and cardia bifida following PODXL morpholino knockdown. After knockdown of PODXL in chick embryos, 34.8% of embryos exhibited normal heart development, 46% showed abnormal cardiac looping, and 15.3% developed cardia bifida.

9. Identification of candidate PODXL regulatory regions using single-cell RNA-seq and ATAC-seq

Previous work in the laboratory generated single-cell RNA sequencing (scRNA-seq) and ATAC-seq datasets from chick embryos during early mesoderm development (Pouncey, 2023). These datasets were analysed to identify candidate cis-regulatory elements associated with PODXL expression.

UMAP visualisation of the scRNA-seq data revealed clusters corresponding to different embryonic tissues, including lateral plate mesoderm (LPM), early somites, and endothelial populations (Fig. 29A). Mapping PODXL transcript expression onto the UMAP showed enrichment within clusters corresponding to lateral plate mesoderm and endothelial cells (Fig. 29B).

ATAC-seq data were then examined to identify regions of accessible chromatin surrounding the PODXL genomic locus. Multiple peaks of chromatin accessibility were detected within approximately 100 kb of the PODXL gene, including peaks associated with lateral plate mesoderm, early somites, and cardiac tissues (Fig. 30). These regions were selected as candidate enhancer elements for further analysis.

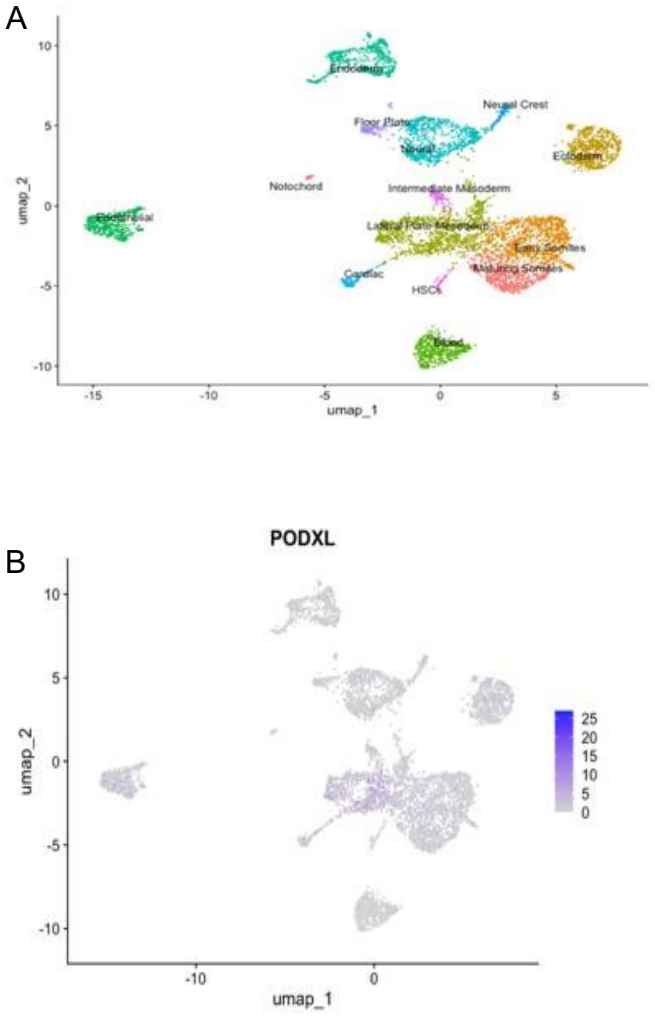

Figure 29. Single-cell RNA-seq analysis identifies PODXL-expressing cell populations

(A) UMAP representation of chick embryo single-cell RNA-seq data showing clusters corresponding to different embryonic tissues including lateral plate mesoderm, early somites, and endothelial cells.

(B) Overlay of PODXL expression on the UMAP demonstrating enrichment in lateral plate mesoderm and endothelial clusters.

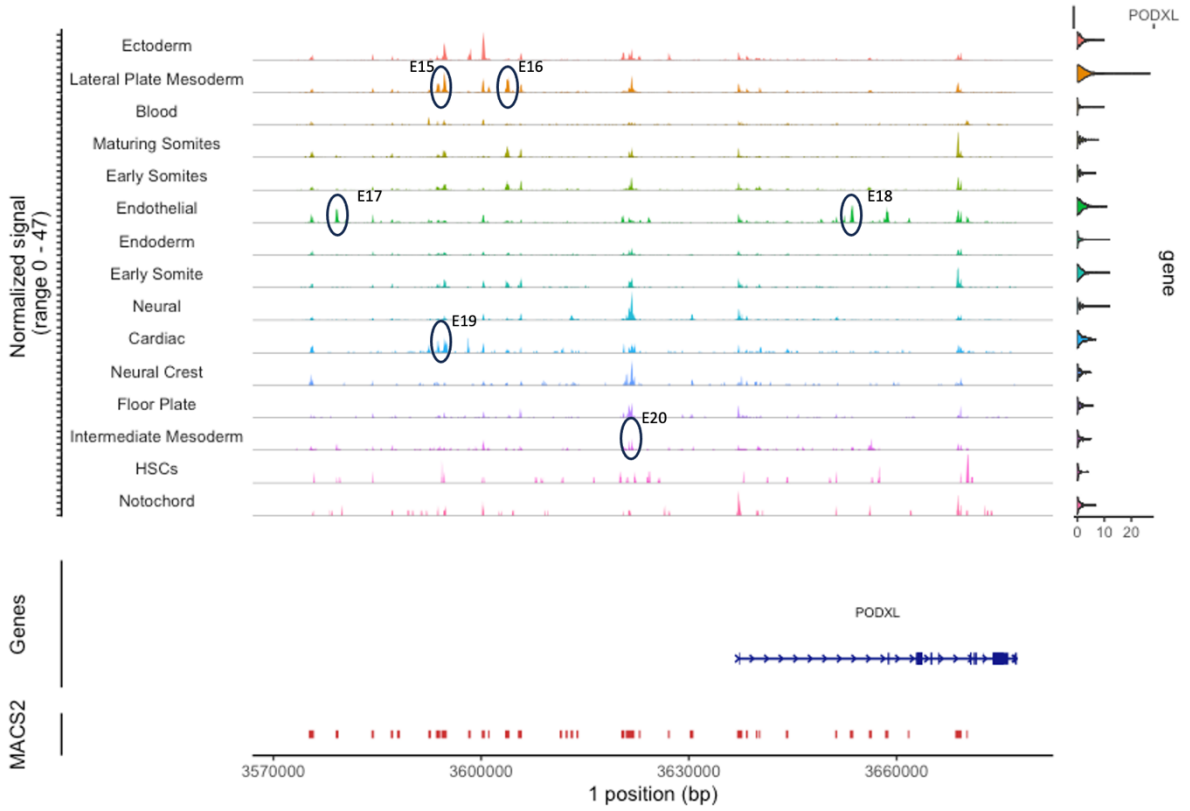

Figure 30 – Chromatin accessibility around the PODXL locus identifying candidate enhancer regions.

ATAC-seq signal tracks showing chromatin accessibility across multiple embryonic tissue clusters surrounding the PODXL gene locus (~100 kb region). Peaks of accessible chromatin indicate regions of open chromatin that may represent potential regulatory elements. Strong signals are observed in several mesoderm-derived tissues, including the lateral plate mesoderm (LPM), endothelial, cardiac and intermediate mesoderm populations. Circled peaks highlight six candidate enhancer regions selected based on chromatin accessibility patterns near the PODXL locus. MACS2 peak calling was used to identify significant regions of accessibility, representing sites that may be available for transcription factor binding and gene regulation. Data adapted from Pouncey, L (2023).

Candidate enhancer sequences identified from ATAC-seq analysis were cloned into a ptk-citrine reporter construct to assess their regulatory activity in vivo. Primers were designed to amplify the candidate enhancer region and the fragment was inserted upstream of a minimal TK promoter driving citrine reporter expression.

Following electroporation of the reporter construct into chick embryos, citrine fluorescence was detected in the lateral plate mesoderm (LPM) (Fig. 31A). Reporter activity was primarily observed in the LPM region, indicating that the candidate enhancer is active in this tissue during early embryonic development.

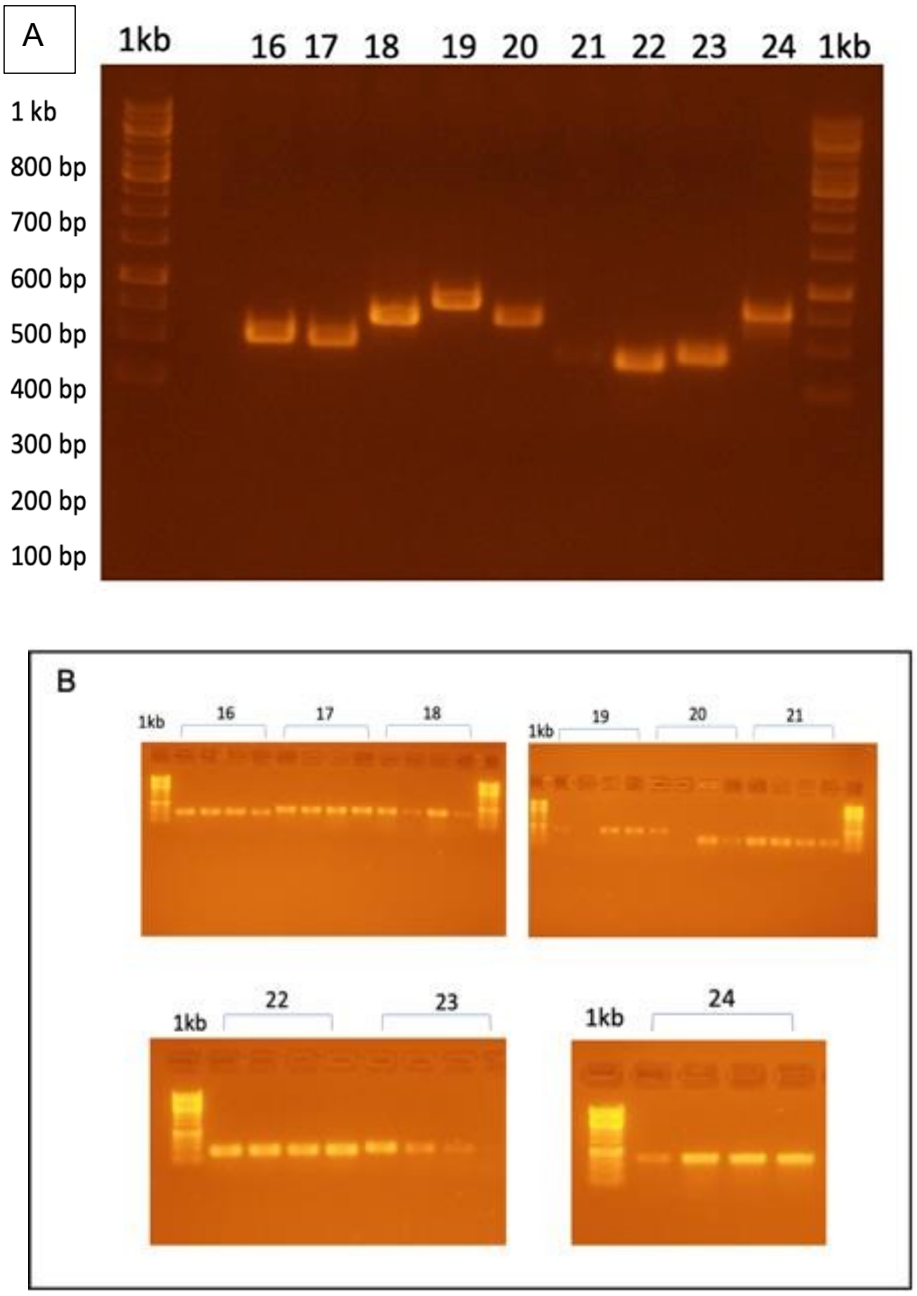

Figure 31 – PCR amplification of candidate PODXL enhancer fragments.
Agarose gel electrophoresis showing PCR products corresponding to candidate PODXL enhancer regions (lanes 16–24). DNA fragments were separated on an agarose gel and visualised using a 1 kb DNA ladder as a molecular weight marker (lanes 1 and 11). Bands correspond to amplified enhancer fragments of approximately 450–550 bp.

Candidate enhancer sequences identified from ATAC-seq analysis were cloned into a ptk-Citrine reporter vector to assess their regulatory activity in chick embryos. Each enhancer fragment was inserted upstream of a minimal thymidine kinase (TK) promoter, which drives expression of the Citrine fluorescent reporter gene when activated by an enhancer element. Figure 32 shows the plasmid construct used for PODXL Enhancer 15, which contains the candidate enhancer region cloned upstream of the TK promoter in the reporter vector.

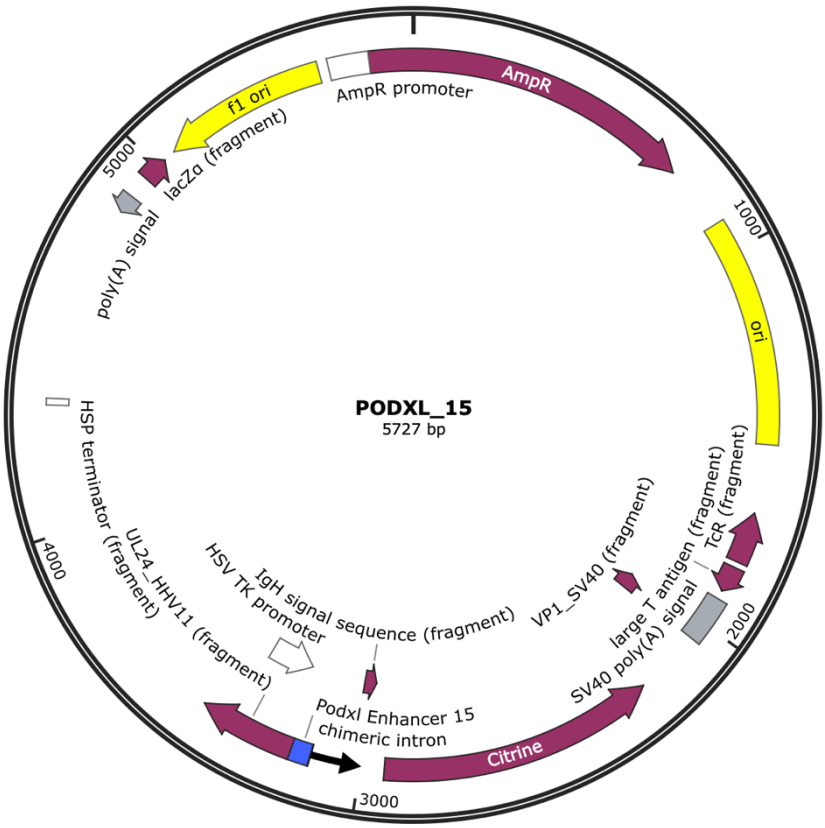

Figure 32. Reporter construct used for testing PODXL enhancer activity.
Plasmid map of the ptk-Citrine reporter construct containing PODXL Enhancer 15 cloned upstream of a minimalthymidine kinase (TK) promoter driving expression of the Citrine fluorescent reporter gene.

9.1 Visualising enhancer- constructs targeting regulatory regions of PODXL using ptk fluorescent reporter system

To test whether the candidate regulatory regions identified from the ATAC-seq analysis were able to drive gene expression, enhancer constructs were generated using a ptk-citrine reporter system. Primers were designed to amplify the candidate enhancer region identified near the PODXL locus, and the amplified fragment was cloned upstream of a minimal HSV TK promoter driving citrine fluorescent reporter expression.
Following electroporation of the reporter construct into chick embryos, embryos were allowed to develop and were then examined for reporter activity. Citrine fluorescence was detected in the lateral plate mesoderm (LPM) of the embryos (Fig. 33A). The localisation of fluorescence to the LPM suggests that this candidate enhancer is active in this region during early embryonic development.

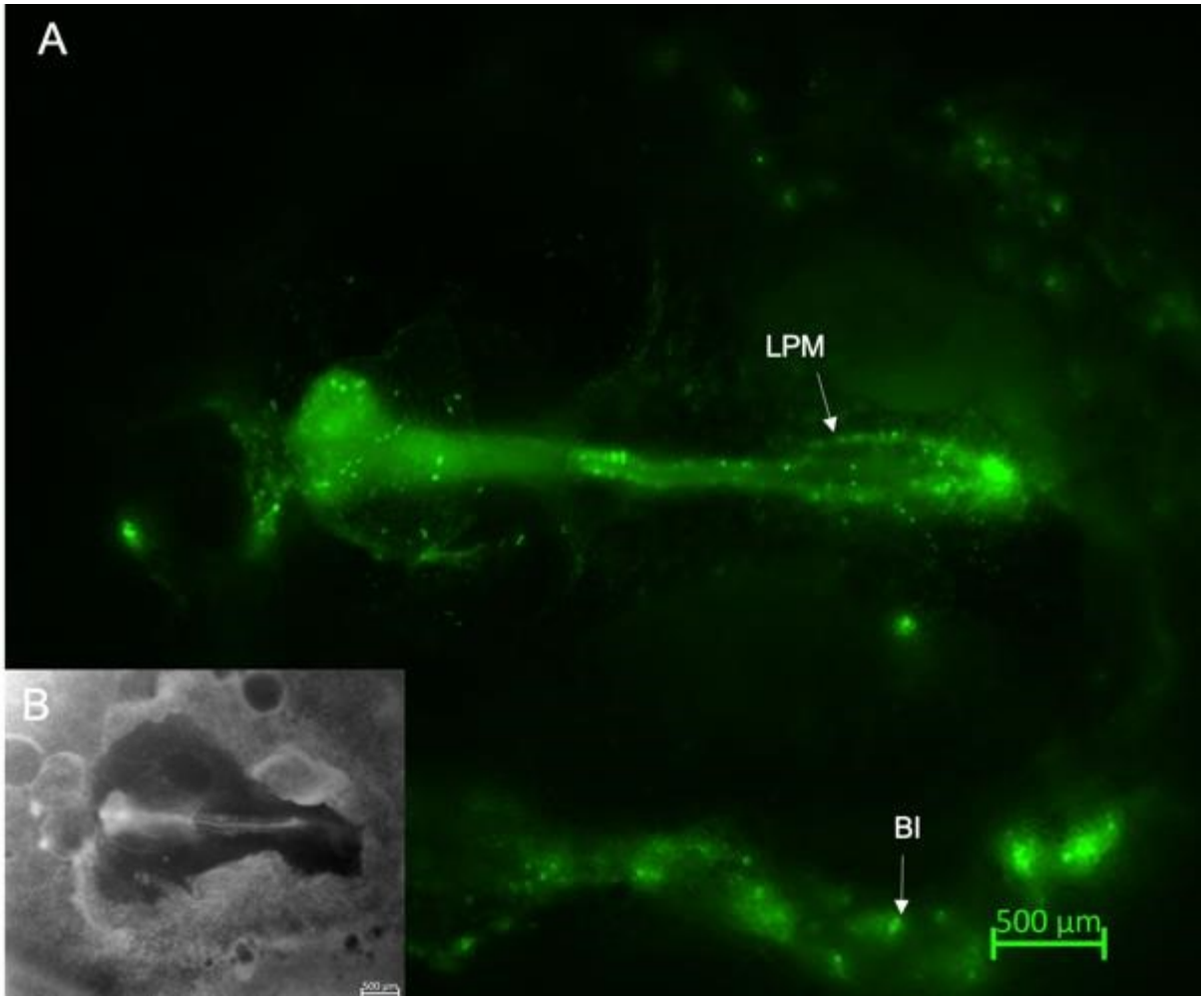

Figure 33 – Reporter activity of PODXL Enhancer 15 in chick embryos.
Chick embryos electroporated with a ptk-citrine reporter construct containing PODXL Enhancer 15 show citrine fluorescence (green) in the lateral plate mesoderm (LPM). This indicates enhancer-driven reporter expression in this tissue during early embryonic development. Representative images are shown (n = 2 embryos showing reporter activity out of 6 embryos injected).
scale bar 500 um.

9.2 Enhancer elements regulating PODXL can be seen in blood islands and endothelial cells.
Embryos were injected with the next candidate enhancer construct, Enhancer 16, to assess its potential regulatory activity during early hematoendothelial development. Preparation of the reporter construct and electroporation procedure were performed as described for Figure 33. To confirm successful injection and electroporation, mCherry (RFP) was included in the injection mix (1 µg/µL). Successful uptake of the construct was shown by the presence of red fluorescence throughout the embryo (Fig. 34B).
Following electroporation, embryos were allowed to develop for a further 24 hours to reach approximately Hamburger–Hamilton stage HH10, when blood islands, endothelial cells, and other hematoendothelial structures begin to form. Embryos were then examined under a Zeiss stereo microscope using a GFP filter set to detect citrine reporter expression.
Green fluorescence was observed in regions corresponding to blood islands and endothelial cells (Fig. 34). This pattern of reporter activity indicates that Enhancer 16 is active in hematoendothelial tissues during early embryonic development, suggesting that it may contain cis-regulatory elements involved in regulating PODXL expression during the emergence of hematoendothelial progenitor (HEP) cells.

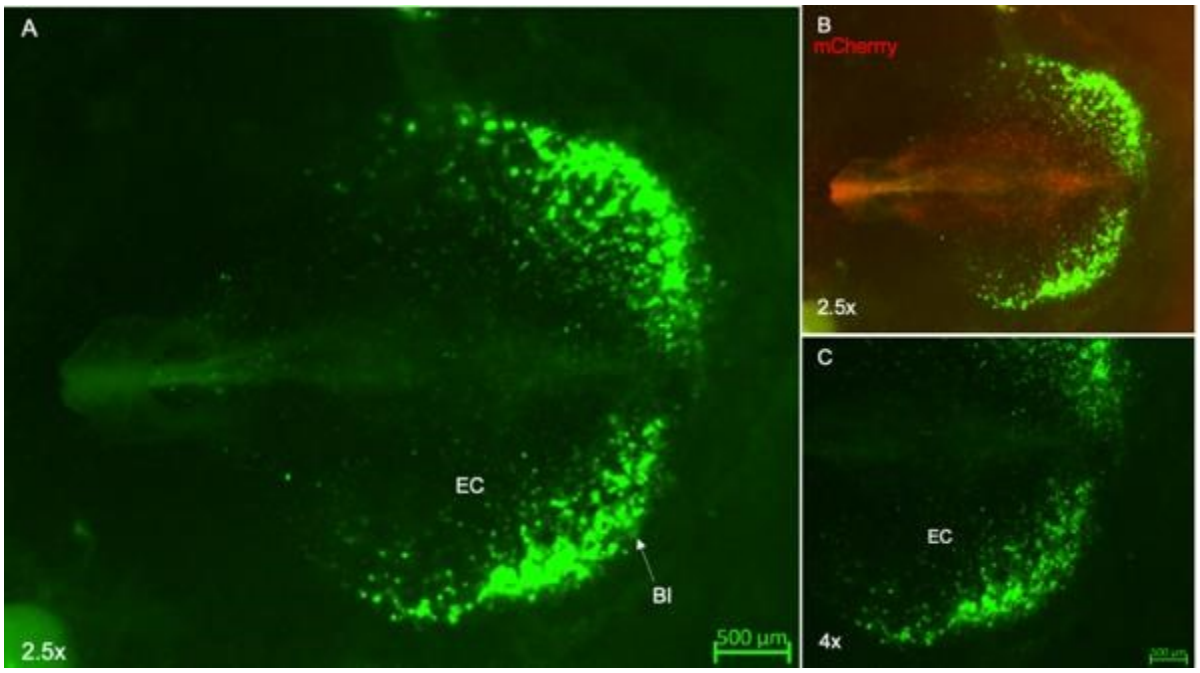

Figure 34 – Reporter activity of PODXL Enhancer 16 in chick embryos.
Chick embryos injected with the Enhancer 16 ptk-citrine reporter construct and imaged using a Zeiss stereo microscope. (A) Image captured at 2.5× magnification showing green fluorescence in the blood island (BI) region and endothelial cells (EC), indicating reporter activity. (B) Image captured under an RFP filter, showing mCherry fluorescence throughout the embryo, confirming successful injection and electroporation. (C) Higher magnification (4×) image of the blood island region showing cells expressing green fluorescence. n = 6 embryos, scale bar = 500 µm.

9.3 PODXL enhancer activity is observed in the dorsal aorta

Embryos were injected with the Enhancer 17 ptk-citrine reporter construct to assess its regulatory activity during early vascular development. Following injection and electroporation, embryos were incubated for a further 24 hours to reach approximately Hamburger–Hamilton stage HH10. Embryos were then examined using a Zeiss stereo microscope with a GFP filter set to detect citrine reporter expression.

Green fluorescence was observed in endothelial cells and in the dorsal aorta (DA) (Fig. 35). This pattern of reporter activity indicates that Enhancer 17 is active in vascular tissues during early embryonic development. The presence of fluorescence in the dorsal aorta suggests that this candidate enhancer may regulate PODXL expression in endothelial cells of the developing vasculature.

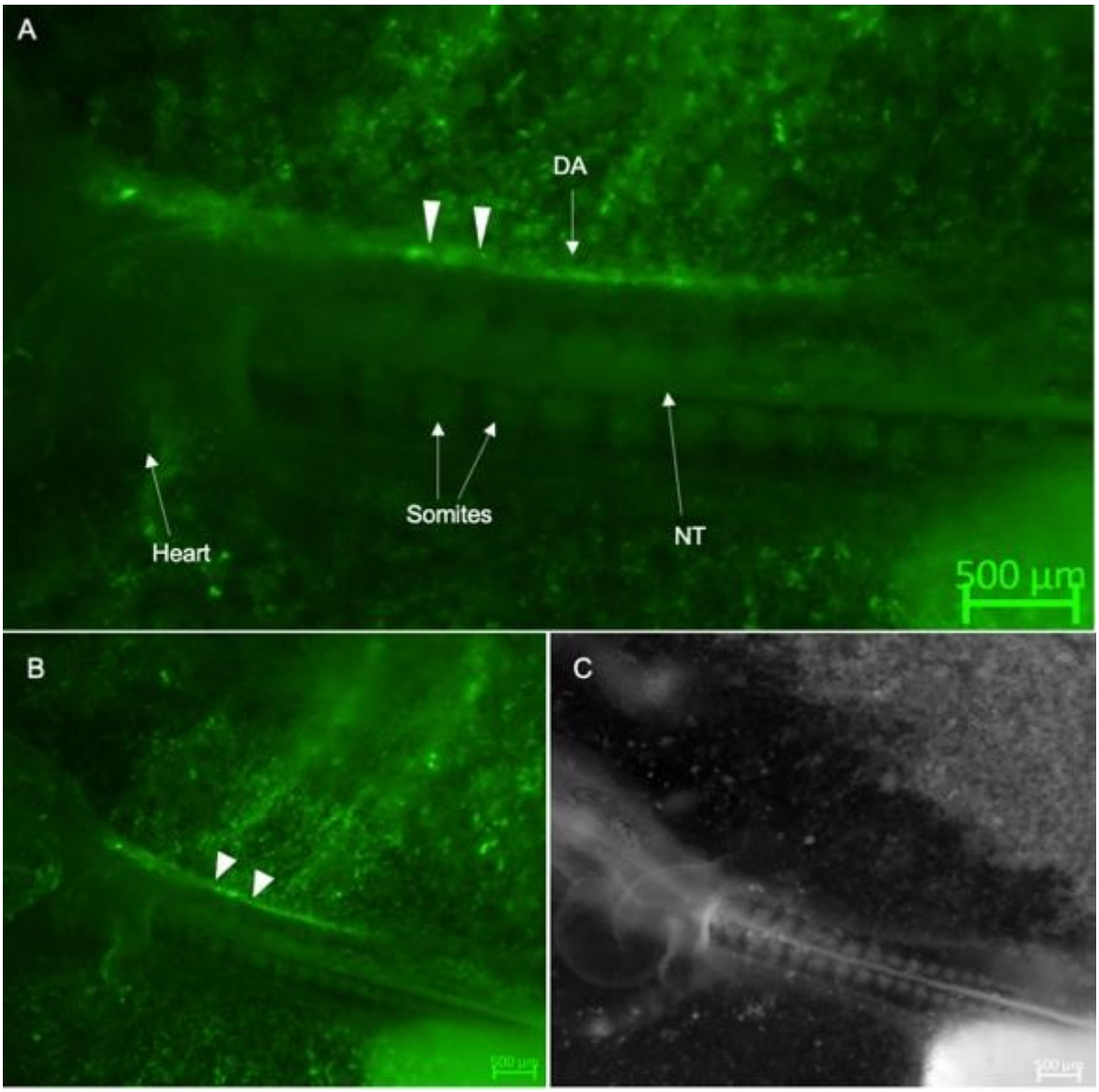

Figure 35- Activity of enhancer region 17 in HH10 chick embryos*. Embryos were injected with enhancer 17 construct and electroporated, followed by 24-hour incubation. (A)Fluorescence was observed using a Zeiss stereo microscope, indicating green fluorescence in endothelial cells and the dorsal aorta (DA) at 8x magnification. (B)4x magnification. (C) 4x magnification, under brightfield. The presence of fluorescence in these regions suggests the enhancer construct targets regulatory elements associated with PODXL expression in the dorsal aorta. This supports the potential role of PODXL in endothelial cell regulation and vascular development.*

9.4 Activity of Enhancer 18 in chick embryos

At Hamburger–Hamilton stage HH10, embryos injected with the Enhancer 18 ptk-citrine reporter construct showed weak green fluorescence in the blood island regions. No clear reporter activity was observed in other mesodermal tissues, including the lateral plate mesoderm (LPM). The fluorescence signal detected in the blood island region was low in intensity compared with the reporter activity observed for other candidate enhancers tested in this study (Fig. 36).

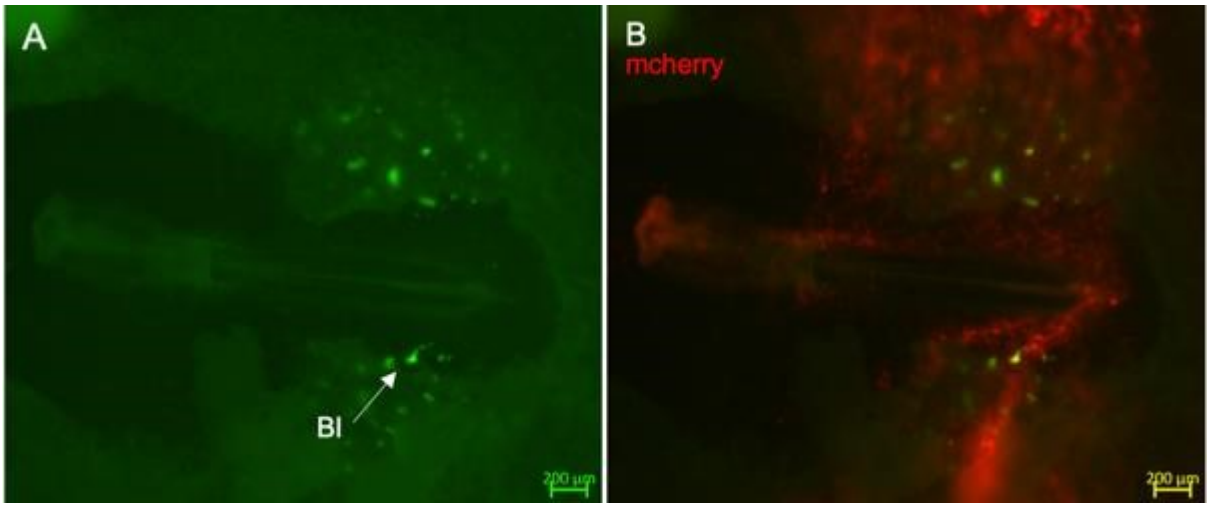

Figure 36 – Reporter activity of PODXL Enhancer 18 in chick embryos.

Chick embryos were injected with the Enhancer 18 ptk-citrine reporter construct at HH3 and electroporated. After a further 24 hours of development to approximately HH10, embryos were imaged using a Zeiss stereo microscope. Weak green fluorescence was observed in the blood island (BI) regions, while no detectable fluorescence was observed in other mesodermal tissues such as the lateral plate mesoderm (LPM). Scale bar = 200 µm.

9.5 Activity of Enhancer 19 in the heart and dorsal aorta

Embryos injected with the Enhancer 19 ptk-citrine reporter construct were allowed to develop to approximately Hamburger–Hamilton stage HH11 before imaging. Embryos were examined using a Zeiss stereo microscope, where green fluorescence was observed in the heart, dorsal aorta (DA), and surrounding endothelial cells (Fig. 37A) at 8× magnification.
Reporter activity in these regions indicates that Enhancer 19 is active in cardiovascular tissues during early embryonic development. Fluorescence in the dorsal aorta and surrounding endothelial cells highlights enhancer activity in regions associated with the developing vascular system.

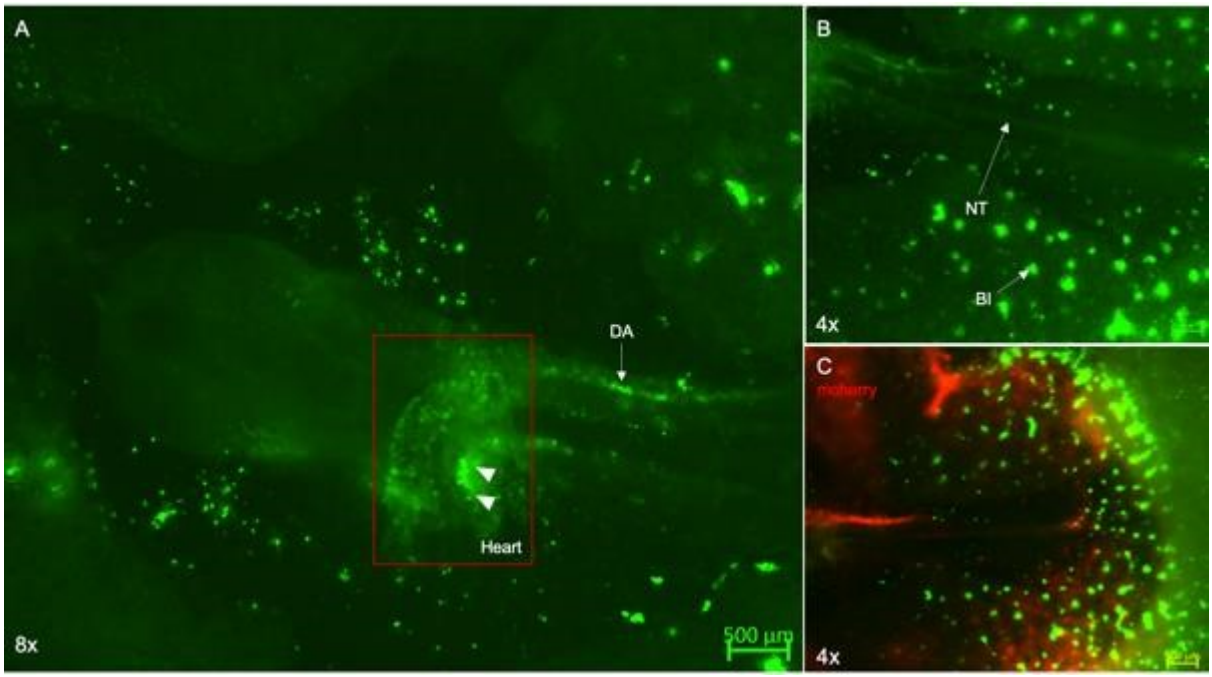

Figure 37 – Reporter activity of PODXL Enhancer 19 in chick embryos.
Chick embryos were injected with the Enhancer 19 ptk-citrine reporter construct at HH3 and allowed to develop for a further 24 hours to approximately HH11. Embryos were imaged using a Zeiss stereo microscope. (A) 8× magnification showing green fluorescence in the heart, dorsal aorta (DA), and surrounding endothelial cells. (B) 4× magnification of the embryo highlighting fluorescence in cardiovascular tissues. (C) 4× magnification showing fluorescence in the blood island region. Scale bar = 500 µm.

9.6 .Activity of Enhancer 20 in cardiac and endothelial tissues

To further assess potential regulatory elements associated with PODXL, embryos were injected with the Enhancer 20 ptk-citrine reporter construct and electroporated at HH3. Embryos were then incubated for approximately 24 hours to allow development to Hamburger–Hamilton stage HH11, when cardiovascular and endothelial structures are clearly visible. Reporter activity was assessed using fluorescence microscopy.

Green fluorescence was detected in the heart and surrounding endothelial cells, indicating enhancer-driven reporter expression in these tissues (Fig. 38).

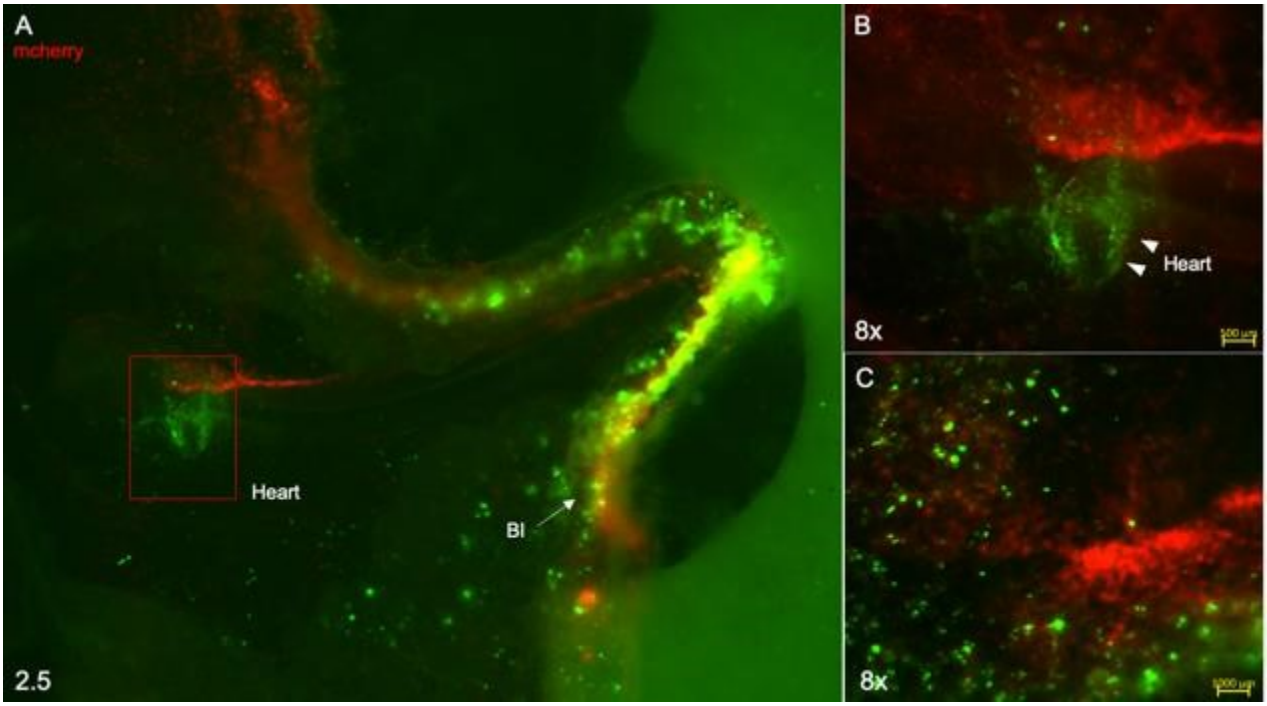

Figure 38 – Reporter activity of PODXL Enhancer 20 in chick embryos

Chick embryos injected with the Enhancer 20 ptk-citrine reporter construct were allowed to develop to approximately HH11 before imaging.

(A) Image captured at 2.5× magnification showing green fluorescence in the heart region, indicating reporter activity.

(B) 8× magnification showing fluorescence in the heart with both GFP (green) reporter signal and RFP (red) indicating successful construct uptake.

(C) 8× magnification showing GFP and RFP fluorescence in endothelial cells.

Green fluorescence indicates enhancer-driven reporter activity.

n = 1 embryo, scale bar = 500 µm.

10. Discussion

In this study, we focused on the development of early hematoendothelial progenitor (HEP) cells and investigated the potential role of PODXL, an extracellular matrix (ECM)-associated glycoprotein, in processes that may influence their differentiation into blood and endothelial lineages. In addition to HEP cells, we also examined PODXL expression in associated cardiovascular structures in the avian chick embryo.

Various approaches were used to gain a more comprehensive understanding of PODXL's role in early HEP development, including gene expression analyses, functional knockdown experiments, and enhancer mapping. Through hybridisation chain reaction (HCR), we mapped the spatial expression profile of PODXL, employed splice-blocking morpholinos to knock down PODXL and assess its potential functional role, and cloned cis-regulatory elements to identify which enhancers may drive PODXL activity. These approaches revealed that PODXL is expressed in mesodermal and vascular territories relevant to hematoendothelial development, that its knockdown interferes with normal cardiac morphogenesis, and that specific enhancer elements show activity in blood islands (BI), lateral plate mesoderm (LPM), and the dorsal aorta (DA).

This investigation therefore aimed to provide insight into how an ECM-associated protein such as PODXL may contribute to early hematoendothelial development and highlights the complexity of PODXL regulation through distinct enhancer elements.

10.1 PODXL and TAL1 expression emerge in the lateral plate mesoderm, where HEP cells arise

Our first step in understanding the potential role of PODXL in HEP development was to use hybridisation chain reaction (HCR) to define the spatial and temporal expression of PODXL relative to Tal1 during early chick development.

Tal1 is a key hematopoietic transcription factor essential for blood and vascular development. Previous studies have shown Tal1 expression in the most posterior lateral domains of chick embryos from HH5-13 (Thierry Jaffredo et al., 2005) and in blood island clusters of angioblasts in the extraembryonic mesoderm (Mink et al., 2003).

This supports our results, as Tal1 expression can be observed in the blood islands at stage HH8 (Fig. 20b). Additional expression was also observed near somite regions within the lateral plate mesoderm (Fig. 20b), suggesting its involvement in vascular development. These observations align with Tal1's established function in early hematopoietic specification (Kallianpur et al., 1994) and its presence in endothelial precursors and angioblast populations. Across stages HH8-11, Tal1 expression was consistently detected in blood islands (Fig. 20b), endothelial cells (Fig. 20b), and the lateral plate mesoderm (Fig. 22b), further supporting its role in progenitor specification.

In contrast, PODXL, a transmembrane glycoprotein known to be expressed in various cell types including vascular endothelial cells and hematopoietic progenitors in mice (Le Tran et al., 2021), exhibited a distinct expression pattern in the heart-forming region (Fig. 22a) and emerging within the lateral plate mesoderm in HH10 and HH11 chick embryos.

embryos (Fig. 22). This demonstrates a broader expression pattern across these tissues.

Merged images (Fig. 28c) showed only partial overlap between PODXL and Tal1 expression in the lateral plate mesoderm (LPM). This indicates that PODXL is not expressed in all Tal1+ cells and may instead be present in neighbouring cells within the LPM. This suggests that PODXL may influence the local microenvironment, potentially affecting cell adhesion or

extracellular matrix organisation, which could indirectly support the development of Tal1+ hematoendothelial progenitors. However, the relationship between PODXL and Tal1 remains unclear, and further experiments would be required to determine whether PODXL acts upstream, downstream, or in parallel to Tal1 during HEP development.

Previous studies have shown that the spatial regulation of PODXL within the glycocalyx is crucial for modulating adhesion and polarity cues, providing a mechanistic basis by which PODXL could shape local tissue environments during development (Román-Fernández et al., 2023).
These results may also suggest that PODXL marks a broader mesodermal population than Tal1, as Tal1 expression is more restricted to hematopoietic regions. Therefore, the observed co-expression may represent a subset of Tal1+ cells arising from a larger PODXL+ mesodermal population that is transitioning toward a hematoendothelial fate. However, this observation alone does not establish whether Tal1 directly regulates PODXL expression or whether Tal1-expressing cells subsequently upregulate PODXL as they commit to hematopoietic lineages.
Previous studies in mouse embryoid bodies and embryonic mesoderm have shown that PODXL expression emerges prior to and then overlaps with Flk1+ populations, allowing Flk1+ mesodermal cells to be separated into two groups: Flk1+PODXL+ cells enriched for hematopoietic potential and Flk1+PODXL− cells that are more likely to give rise to endothelial or cardiac progenitors (Zhang et al., 2014). This suggests that PODXL can distinguish between progenitor populations with different developmental potentials rather than simply marking broad mesoderm.
In the chick embryo, the broader distribution of PODXL observed in this study may indicate that PODXL marks a wider pool of mesodermal cells, of which only a subset later becomes Tal1+ hematoendothelial progenitors. The cardiac and mesodermal defects observed following PODXL knockdown in this study support this possibility, suggesting that disruption of PODXL may affect the wider mesodermal environment from which cardiac and vascular progenitors arise.
To further investigate the spatial and temporal expression of PODXL, we performed additional HCR experiments using Tal1 and PODXL probe sets and imaged embryos at higher magnification (10x) using confocal microscopy (Fig. 24). This allowed us to observe PODXL expression emerging from the paraxial mesoderm (Fig. 29a), while Tal1 expression remained largely restricted to the posterior lateral plate mesoderm (Fig. 24b). Previous preliminary in situ hybridisation results (Fig. 19b) had suggested that PODXL mRNA was present near the somite or paraxial mesoderm regions. The HCR experiments performed here confirm this observation, further supporting the idea that PODXL is expressed within early mesodermal tissues involved in vascular and hematopoietic development.
Overall, HCR revealed PODXL expression emerging from the paraxial mesoderm, heart-forming region, and lateral plate mesoderm (Fig. 21a, 23a, 24a), while Tal1 expression remained largely confined to the lateral plate mesoderm (Fig. 20b, 21b, 24b). Although partial spatial overlap between PODXL and Tal1 was observed (Fig. 22c), PODXL expression was broader, suggesting that it may play a role in early mesodermal organisation rather than directly controlling hematopoietic specification. Unlike studies in mouse models where PODXL expression has been associated with hematopoietic commitment (Zhang et al., 2014), the findings from this study do not indicate a clear regulatory relationship between PODXL and Tal1 during early chick development. Instead, PODXL expression in mesodermal regions important for vascular and cardiac development suggests that it may contribute to shaping the microenvironment that supports hematoendothelial progenitor emergence.

HCR was also performed on HH4–5 chick embryos to examine whether PODXL or Tal1 expression could be detected at earlier developmental stages. In these experiments, no PODXL or Tal1 expression was observed at HH4 or HH5, suggesting that both genes may become activated later during mesoderm differentiation rather than during the earliest stages of mesoderm formation.

The absence of detectable PODXL and TAL1 expression at HH4–5 may indicate that both genes are activated later during development, potentially in response to signalling pathways that promote lineage commitment toward hematopoietic or endothelial cell fates. Several signalling pathways are known to regulate these early developmental processes. For example, BMP and WNT signalling pathways have been shown to play important roles in mesodermal patterning and the specification of hemangioblasts populations (Nostro et al., 2008). In addition, Notch signalling is known to regulate hematopoietic differentiation, while VEGF signalling is critical for endothelial lineage specification. The delayed expression of PODXL and TAL1 observed in this study may therefore reflect the stage at which these signalling pathways begin to influence hematoendothelial progenitor emergence.
To further investigate the spatial and temporal regulation of PODXL and TAL1, additional experiments could be used to quantify gene expression levels during early development. For example, quantitative PCR (qPCR) could be used to measure relative mRNA levels across developmental stages (HH4–HH11). An advantage of this approach is its high sensitivity, allowing precise quantification of transcript abundance over time. However, a limitation of qPCR is that spatial information is lost, meaning that the method would reveal overall expression levels but not the specific embryonic regions where the genes are expressed.
Alternatively, the fluorescence intensity of PODXL and TAL1 signals obtained through HCR imaging could be quantified using software such as Fiji/ImageJ, allowing comparison of relative expression levels between developmental stages. This approach would retain spatial information while providing semi-quantitative insight into changes in expression patterns during development.

10.2 Knockdown of PODXL through splice-blocking morpholinos had no effect on TAL1 expressing cells.

Having established the spatial and temporal expression patterns of PODXL and Tal1 through hybridisation chain reaction (HCR), we proceeded to investigate their functional roles in haematopoiesis. PODXL and TAL1 expression patterns can be seen emerging from the lateral plate mesoderm (Fig.) and to further support our hypothesis that ECM molecules are involved in hematoendoelial progenitor development, we conducted knock-down experiments aimed at assessing the impact of PODXL knockdown on haematopoiesis and Tal1 expression. Splice blocking morpholinos (fig) were employed to effectively reduce PODXL levels and aimed to elucidate whether this reduction would disrupt hematopoietic development. A splice-blocking morpholino works by targeting exon–intron junctions, resulting in mis-spliced transcripts and loss of functional protein. This allows knockdown efficiency to be validated by RT-PCR through detection of changed transcript sizes. Translation-blocking morpholinos were not used as they prevent protein synthesis without degrading mRNA, making experimental validation more difficult.

Following the knockdown, HCR was conducted for TAL1 to determine whether disruption of PODXL expression had any downstream effects and if it would indicate a direct link between these two molecules in regulating hematopoietic development. We observed *Tal1* expression emerging from the LPM, suggesting that haematopoiesis is not affected (fig 24a). However, to strengthen my investigation, I could have included samples from embryos that have not undergone PODXL knockdown as a control. This will allow me to compare the expression levels of TAL1 in both knock-down and control embryos. This can be achieved by quantifying the fluorescence intensity of TAL1 signals and statistically analysing the difference in expression levels.

10.3.1 Knockdown of PODXL has a role in cardiac development

The embryos were then observed under Zeiss stereo microscope. Interestingly, while there was no effect on TAL1 expression, suggesting no effect on haematopoiesis, 46% of the embryos injected developed abnormal heart looping and 15.3% of injected embryos developed cardia bifida, while 38.4% presented with wildtype development (Fig. 28). This raises important questions regarding the functional relationship between PODXL and heart development.

Heart tube development begins with the migration of presumptive heart cells between the ectoderm and endoderm toward midline, guided by fibronectin gradients in the foregut endoderm Farraj and Zeltser (2021). This migration halts when the cells reach the lateral walls of the anterior gut tube, where they form the two heart primordia. As the primordia merge to form a single heart tube, cardiac looping establishes the correct heart architecture. While ECM distribution does not have an instructive role in heart looping, it plays a critical function in guiding earlier developmental events. For example, early inhibition of ECM-remodeling enzymes like MMP2 results in heart migration defects, including cardia bifida, a condition where the heart fails to fuse at the midline (del Monte-Nieto et al., 2020)

Disruptions in this process, such as those caused by PODXL knockdown, can result in abnormal looping (Fig. 25,26,27) and cardia bifida (fig. 27), as seen in my experiments. As proper heart function is important for creating a supportive hematopoietic niche, these cardiac defects could impact haematopoiesis indirectly despite TAL1 expression being unaffected.

In cardiac development, PODXL is highly expressed in endothelial cells, where it helps prevent non-specific adhesion and supports the structural integrity of capillaries (Le Tran, Wang and Nie, 2021). The findings from the morpholino knockdown experiments could suggest that PODXL plays a role in the adhesion and migration of cells during cardiac morphogenesis.

PODXL's functions in cell adhesion and migration could be important for the proper migration of cardiac progenitors to form the heart tube. Additionally, signalling pathways such as the Nodal-pitx2 pathway, are crucial for Left-right asymmetry and could have been impacted by knockdown of PODXL. The Nodal-pitx2 pathway is established in lateral mesoderm tissue (Kajikawa, E 1998). Furthermore, while TAL1 expression and haematopoiesis were unaffected, other ECM molecules like CD34 Darina Bačenková et al. (2025) might be responsible in compensating haematopoiesis regulation. Despite this, figures 25,26,27

suggest that PODXL plays a role in cardiovascular development which could impact hematopoietic niches at later stages. Western blots can be performed to detect and quantify protein levels of PODXL, by using specific antibodies to detect the PODXL protein. We can also perform RT-qPCR to allow for precise quantification of how much PODXL is expressed and determine whether PODXL is downregulated by comparing samples between knockdown and control embryos. Control embryos can be injected with a scrambled morpholino that does not target PODXL gene.
My results revealed that while Tal1 expression was restricted to the blood islands and posterior LPM, PODXL was detected in the paraxial mesoderm and heart-forming region, with partial overlap in the LPM. These findings suggest that PODXL may contribute to mesodermal patterning rather than directly regulating Tal1+ hematopoietic progenitors. The confirmation of PODXL expression in early mesoderm further supports its potential role in influencing the microenvironment where Tal1expressing progenitors emerge. Further investigation using lineage tracing or molecular profiling of PODXL+ mesodermal cells would help in identifying its precise role in hematopoietic specification.

The tissue-specific regulatory activity of PODXL's enhancer regions further supports its dual role in cardiovascular and hematopoietic development. The enhancer cloning results, which showed fluorescence localized to the lateral plate mesoderm, dorsal aorta, endothelial cells and cardiac regions align with the gene's known expression in endothelial cells and HSCs. These findings suggest that PODXL is a critical ECM molecule involved in both cardiovascular development and haematopoiesis, needing further research into its regulatory mechanisms and interactions with other ECM components.

10.3.2. Investigating the role of PODXL in cell adhesion and migration

The cardiac defects observed following PODXL knockdown suggest that PODXL may play a role in regulating cell adhesion or migration during early embryonic development. As PODXL is known to interact with cytoskeletal regulators and adhesion molecules (Fernández et al., 2013), disruption of its expression could affect the ability of mesodermal cells to migrate properly during heart tube formation.

To investigate whether PODXL directly influences cell migration, live imaging experiments could be performed in chick embryos. For example, mesodermal cells in the lateral plate mesoderm could be labelled with fluorescent reporters and tracked during development in both control and PODXL knockdown embryos (Sweetman et al., 2008; Kusumoto et al., 2016). Looking at the direction, speed, and coordination of cell movements of both groups would allow us to see whether PODXL disruption alters normal migratory behaviour.

Additionally, cell adhesion assays could be performed to determine whether PODXL influences the ability of cells to adhere to extracellular matrix substrates. Cells isolated from chick embryos or cultured endothelial cells expressing PODXL could be plated on ECM components such as fibronectin or laminin, and adhesion strength could be quantified by measuring the number of cells remaining attached after washing. Comparing adhesion properties between control and PODXL knockdown conditions would help determine whether PODXL contributes to adhesion-mediated signalling during development, as it has been previously shown to regulate cell adhesion and migration (Larrucea et al., 2008).

Immunostaining for actin cytoskeleton components following Podxl knockdown can help to examine changes in cytoskeletal organisation. It could reveal whether disruption of Podxl affects cell polarity or tissue organisation within the developing mesoderm. These experiments can also help to determine whether cardiac defects are due to impaired cell migration, altered adhesion, or broader disruptions to mesodermal tissue development.

10.4. Cis-Regulatory Mechanisms of PODXL in Hematopoietic and Cardiovascular Development

Following the observation that functional disruption of PODXL resulted in cardiac abnormalities, we next investigated how PODXL may be regulated at the transcriptional level. To address this, we examined the potential cis-regulatory elements controlling PODXL expression using enhancer cloning approaches.

Enhancers are regulatory DNA elements that control when and where genes are expressed during development. They function as binding sites for transcription factors and allow precise spatial and temporal regulation of gene expression. In this study, we aimed to determine whether potential enhancer regions associated with PODXL were active in tissues relevant to hematoendothelial development. If enhancer activity was observed in the lateral plate mesoderm, where TAL1 is expressed and where hematoendothelial progenitors arise, this would suggest that these regulatory regions may contribute to controlling PODXL expression during early development.

ATAC-seq data (Fig. 29,30) were used to identify regions of accessible chromatin surrounding the PODXL locus, and several candidate enhancer regions were selected for cloning and linked to a reporter construct. Enhancer constructs were generated using a Ptk-citrine reporter system. Reporter activity revealed that several of the identified enhancer regions drove GFP expression in mesoderm-derived tissues including the lateral plate mesoderm (Fig. 33), dorsal aorta (Fig. 37B), endothelial cells (Fig. 34A), and cardiac regions (Fig. 38A, Fig. 37A).

The presence of enhancer activity across multiple mesoderm-derived tissues suggests that regulation of PODXL expression may occur broadly within mesodermal lineages rather than being restricted only to hematopoietic progenitor specification.

10.4.1. Lateral Plate Mesoderm (LPM)

Enhancer activity was observed in the lateral plate mesoderm (Fig.23,24), suggesting that PODXL expression may be regulated in this region. These observations align with the HCR results presented earlier (Fig.23B), which showed PODXL and Tal1 expression emerging from or adjacent to the LPM.

The lateral plate mesoderm is an important embryonic region that gives rise to cardiovascular tissues, blood progenitors, and other mesoderm-derived organs (Prummel, Nieuwenhuize and Mosimann, 2020). Therefore, enhancer activity in this region may indicate that PODXL regulation is linked to early mesodermal developmental programs.

Previous studies have suggested that transcriptional regulation in the LPM may be conserved across chordates (Prummel et al., 2019). Although conservation of the candidate PODXL

enhancers was not directly examined in this study, the observed enhancer activity in the LPM suggests that PODXL expression may be controlled by regulatory mechanisms involved in early mesoderm patterning.

10.4.2. Dorsal Aorta (DA)

The dorsal aorta is a key site for hemogenic endothelial cells and hematopoietic stem cell (HSC) emergence around HH12 through the endothelial-to-hematopoietic transition (EHT) (de Bruijn et al., 2000). In this study, enhancer activity was observed in the dorsal aorta (Fig.35), suggesting that regulatory elements associated with PODXL may be active in this vascular structure.

The presence of enhancer activity in the dorsal aorta suggests that PODXL regulation may begin prior to the emergence of definitive hematopoietic stem cells. PODXL is known to play roles in cell adhesion and polarity, which contribute to endothelial organisation and vascular integrity. These functions may help support the structural environment required for hemogenic endothelial cells. However, the enhancer activity observed in this region does not confirm a direct role for PODXL in HSC specification. Instead, it may reflect a broader function of PODXL in vascular development and endothelial organisation.

10.4.3. Endothelial Cells

Enhancer activity was also observed in endothelial cells (Fig.34), further supporting the association of PODXL with vascular tissues. Endothelial cells form the lining of blood vessels and play a critical role in establishing a functional circulatory system.

During early chick development, blood islands emerging around HH8 give rise to endothelial and hematopoietic cells, which later contribute to the formation of the primitive vascular plexus. This early vascular network supports the transport of oxygen and nutrients during embryonic development.

The enhancer-driven expression observed in endothelial cells may indicate that PODXL expression is regulated in vascular tissues during early development. However, it is also possible that the enhancer activity reflects upstream developmental signalling pathways involved in endothelial specification, including BMP, Notch, Wnt, Sonic hedgehog and TGF-β signalling pathways. These signalling pathways regulate endothelial differentiation and hematopoietic development and may activate PODXL expression indirectly.

10.4.4. Cardiac Region

Enhancer activity observed in cardiac regions (Fig.37, 38) together with the cardiac defects observed following PODXL knockdown (Fig.26, 27) supports the idea that PODXL may play a role in cardiovascular development.

Failure of myocardial precursor migration during early development can lead to cardia bifida, a condition in which two separate heart structures form instead of a single heart tube. Similar phenotypes have been described in zebrafish mutants such as *natter*, which results from disruption of fibronectin expression during myocardial precursor migration (Trinh and Stainier,

2004). Additionally, fibronectin knockdown using antisense morpholinos has also been shown to produce cardia bifida phenotypes (Matsui et al., 2007).

These observations suggest that disruption of proteins involved in cell adhesion or extracellular interactions can affect cardiac precursor migration. The cardiac defects observed following PODXL knockdown in this study may therefore reflect disruption of similar processes during heart development.

10.4.5 Limitations

The chick embryo has significant experimental advantaged for studying early development, including accessibility for manipulation and live imaging, but it also poses some limitations compared to other organisms. Unlike mouse models, stable genetic knockouts and lineage tracing approaches are less well established in chick embryos, limiting the ability to perform long-term genetic analyses. Similarly, zebrafish offer powerful transgenic and high-throughput genetic screening capabilities that are more difficult to implement in avian systems. Nevertheless, the chick remains an important model for investigating early embryonic patterning and hematoendothelial development due to its accessibility and the ability to perform targeted manipulations such as electroporation and morpholino-based gene knockdown (Pouncey & Mok, 2025).

Another limitation of this study is that additional controls were not included for the morpholino experiments. The far- red tag (Lissamine) was used to identify successful uptake of the morpholino, but further controls were not carried out due to time constraints, technical difficulty of microinjecting embryos and high embryo mortality during injections. Using a scrambled MO would have strengthened future experiments

10.4.6. Future investigation of PODXL regulatory elements

To further investigate the regulatory role of these enhancer regions, future experiments could involve CRISPR-Cas9 mediated deletion or mutation of candidate enhancer sequences. Targeted deletion of these regions would allow assessment of whether removing specific enhancer elements affects PODXL expression during development.

For example, guide RNAs could be designed to target the candidate enhancer regions identified in this study. Following CRISPR-mediated deletion, PODXL expression could be assessed using reporter assays or HCR to determine whether these regulatory regions are required for normal gene expression.

In addition, identifying transcription factors that bind to these enhancers would help reveal the regulatory network controlling PODXL expression.

Several transcription factors known to regulate hematopoietic and endothelial development may interact with these regulatory regions. For example, TAL1 and GATA2 are essential regulators of hematopoietic specification and are known to bind enhancer regions in endothelial and mesodermal lineages. TAL1 is expressed in hemangioblasts and hemogenic endothelium, where it regulates genes involved in blood and vascular development. GATA2 is a zinc-finger transcription factor required for the maintenance and function of hematopoietic stem and progenitor cells and plays an important role in endothelial-to-hematopoietic transition (Kang et al., 2018).

In addition, transcription factors such as FOXC1 and FOXC2 are important regulators of cardiovascular development. These factors regulate signalling pathways involved in heart formation and mutations in these genes can lead to congenital heart defects (Seo and Kume, 2006). Investigating whether these transcription factors interact with PODXL regulatory elements may provide further insight into how PODXL expression is controlled during cardiovascular and mesodermal development.

11. Conclusions

This investigation set out to study the role of PODXL, an extracellular matrix-associated protein, in hematoendothelial progenitor (HEP) development using chick embryos. HCR analysis highlighted Tal1 expression in blood islands, the lateral plate mesoderm (LPM), and endothelial precursors, which aligns with its known role in haematopoiesis. PODXL expression was broader, detected in the paraxial mesoderm, heart-forming regions, and LPM, with partial overlap with Tal1. This suggests that PODXL may act upstream of or in parallel with Tal1, possibly influencing the microenvironment required for hematopoietic emergence rather than directly driving lineage commitment.

Morpholino-mediated knockdown resulted in cardiac abnormalities, including cardia bifida and abnormal heart looping, suggesting a role for PODXL in cardiac morphogenesis and possibly in cardiac progenitor migration. However, Tal1 expression following PODXL knockdown was still observed in the LPM, indicating that early Tal1 expression and hematopoietic specification may not be directly dependent on PODXL at these developmental stages. Instead, given PODXL's known role in cell adhesion and migration, it may influence processes such as cardiac progenitor migration and heart morphogenesis.

Identification of cis-regulatory elements surrounding the PODXL locus using ATAC-seq data revealed enhancer regions capable of driving tissue-specific expression in the lateral plate mesoderm, dorsal aorta, endothelial cells, and cardiac regions. These findings provide insight into the cis-regulatory control of PODXL during early mesodermal and vascular development.

Future experiments could include lineage tracing of PODXL+ cells to determine their contribution to hematopoietic versus endothelial lineages. Additional experiments such as double knockdowns of PODXL with key regulators including Tal1 or Gata2 could further clarify their potential interactions. This could involve co-injecting splice-blocking morpholinos with lissamine tags to verify uptake, alongside single knockdown and mismatch control experiments. CRISPR-based deletion of candidate enhancer regions could help validate the functional role of the identified regulatory elements. Incorporating chromatin-based assays may also help clarify how PODXL is positioned within the transcriptional network controlling HEP development.

Overall, this study demonstrates that PODXL is expressed in key mesodermal tissues including the lateral plate mesoderm, paraxial mesoderm and cardiac regions. Disruption of PODXL expression resulted in developmental defects such as cardia bifida and abnormal heart looping, while enhancer analysis revealed regulatory elements that drive PODXL expression in hematoendothelial and cardiac domains. Together, these findings suggest that PODXL may contribute to the organisation of mesodermal environments involved in

cardiovascular and hematoendothelial development. Future studies investigating PODXL and its regulatory elements will help clarify its mechanistic role during embryonic development.

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