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Single-cell Trajectories and Pseudo-time Analysis Reveal Additional Insights into Rheumatoid Arthritis Disease Progression

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

As the chicken embryo grows, its increasing metabolic demand drives the development of the cardiovascular system, enabling efficient nutrient delivery through complex molecular and structural formation. Key extracellular matrix (ECM) components, such as hyaluronic acid (HA) and collagen type I alpha 1 (COL1A1), play a crucial role in shaping the biomechanical environment of the heart, particularly the sinoatrial node (SAN). These ECM elements regulate cell adhesion, migration, and the maturation of cardiac progenitor cells (CPCs), essential for the heart's electrical conductivity and rhythmicity. Based on the hypothesis that ECM mechanical properties influence the spatial patterning of conduction-related genes, we analyzed spatial transcriptomics data from chicken hearts at different developmental stages. Collagen-related genes associated with a sturdy ECM showed an increasing trend over time, while soft ECM-related genes responsible for producing

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proteoglycans and breaking down collagen decreased. Although conduction-related genes did not fully align with ECM trends, intriguing patterns were observed, pointing to potential interactions between ECM remodeling and the construction of the cardiac conduction system. Our findings suggest significant ECM r7emodeling between days 4 and 7, which may influence the heart's structural and functional development.

Keywords: Extracellular matrix; hyaluronic acid; pseudo-time analysis; rheumatoid arthritis; single-cell trajectories.

1. INTRODUCTION

As the chicken embryo grows, its increasing metabolic demand makes the diffusion of nutrients insufficient, prompting the development of the cardiovascular system. This system, the first to form, ensures proper nutrition for the embryo through complex molecular communication and structural formation. Cardiac cells begin to assume their functions based on their spatial position within the heart layers.

During embryonic heart development, cardiac neural crest cells (CNCs) play a crucial role in forming the conduction system and vascular structures. CNCs migrate to both the arterial and venous poles [1,2] of the heart, contributing to the outflow tract septum, smooth muscle cells in vessel walls, and ganglionic cells in the subepicardial space [3,4,5]. These ganglionic derivatives form the anterior parasympathetic plexus (APP), while CNC cells at the venous pole surround the developing conduction system and atrioventricular (AV) cushions, highlighting their role in establishing the heart's pace-making and conduction pathways [6,7,8]. In parallel, extracellular matrix (ECM) remodeling is vital for structural development. The epicardium induces apoptosis in the myocardium [9], which is critical for the proper outgrowth of coronary arteries and their integration into the aorta [10]. Endocardial cushions, essential for forming heart valves, undergo epithelial-to-mesenchymal transformation (EMT), a key ECM-related process. Additionally, pre-valve leaflet formation begins in the AV cushion mesenchyme, dependent on ECM remodeling and cellular processes such as proliferation and differentiation [11]. Early trabecular bundles in the un-septated ventricle further reflect the structural reorganization necessary for later conduction system development.

As the heart progresses into later developmental stages, significant changes occur in both its structure and function. By stage HH 31, apoptosis plays a crucial role in shaping the heart, particularly in areas between the aorta and pulmonary artery and around the AV node and bundle of His [2,12]. This apoptosis is linked to the differentiation of the cardiac conduction system [13], with cardiac neural crest cells (CNCs) contributing to the final formation of the sinoatrial (SA) node, AV node, bundle branches, and the Purkinje fiber network. These cells help establish the electrical pathways required for coordinated contraction of the heart. By stage HH 36, the Purkinje fiber network undergoes significant differentiation, with conductive cells forming around developing coronary vessels [14,15,16,17,18]. This close association between the coronary vasculature and the conduction system highlights the interdependence of these structures in heart maturation.

CNCs also complete their contributions to outflow tract septation by stage HH 31, ensuring proper separation of the aorta and pulmonary artery [5]. By stage HH 36, cardiac innervation becomes well-established, with CNCs differentiating into ganglion cells in the anterior and posterior cardiac plexuses, further refining the heart's ability to regulate its rhythm [7]. The coronary vasculature also matures during this period, with the coronary vessels adopting a regular pattern within the myocardium. These vessels support the developing conduction system, as proper vascular development is essential for the recruitment of conductive myocytes.

Valve development progresses throughout these stages, with the aortic and pulmonary valve leaflets shifting into an angled position relative to each other by HH 31 [19]. The atrioventricular (AV) valves are fully formed by HH 36, although they continue to undergo remodeling and maturation [20,21]. The mitral valve leaflets, composed primarily of mesenchymal cells and extracellular matrix (ECM), form from endocardial cushion tissue, while the tricuspid valve, in the form of a muscle band, incorporates contributions from both endocardial cushions and ventricular myocardium. This dual origin underscores the complexity of valve development [20].

By stage HH 40, the heart's innervation by CNCs is complete, and these cells contribute to nerve tissue in the adventitia of large veins and coronary arteries [7]. The coronary vasculature continues to support the differentiation of the Purkinje fibers, which now extend throughout the sub-endocardium and enable synchronized ventricular contractions [14,16,17,22]. This marks the culmination of heart maturation, with the conduction system and coronary vasculature fully integrated to ensure efficient cardiac function.

Several key genes, such as those responsible for hyaluronic acid (HA), and collagen type I alpha 1 (COL1A1) are of particular interest due to their role in ECM composition and the mechanical properties of the sinoatrial node (SAN) during development. HA-based matrices provide a low-shear, high-viscoelastic environment that supports SAN development, while COL1A1 and TNC contribute to ECM structure, influencing cell adhesion and migration. These genes regulate the ECM's biomechanical environment, promoting cardiac progenitor cell (CPC) maturation and ensuring the heart's electrical conductivity and rhythmicity. Understanding their role in SAN development is critical for research into cardiac pathophysiology.

Building on this foundation, I developed a hypothesis based on a recent study by Henley et al. [23], which examined the relationship between ECM mechanical strength and pacemaker cell activity in the atria of early embryos. My hypothesis suggests that conduction-related genes would display a spatial pattern similar to ECM-related genes. As the heart matures, the ECM stiffens, and the collagenous matrix supports the development of fast-conducting myocardium, while the softer ECM in immature hearts differentiates into various cell types.

To test this hypothesis, I used data from a spatial transcriptomics study on chicken hearts at different developmental stages by Mantri et al. [24]. The study collected transcriptomic information from 5 hearts at day 4, 4 hearts at day 7, 3 hearts at day 10, and 1 heart at day 14, with the data publicly available through the Gene Expression Omnibus (GEO). I analyzed this data to determine whether ECM-related genes exhibit spatial-temporal patterns that correspond to the expression patterns of conduction-related genes.

For the analysis, I classified ECM-related genes into two categories: collagen-related genes, associated with a sturdy ECM with high Young's modulus, and soft ECM-related genes, involved in producing proteoglycans and breaking down collagen. The study revealed an opposing trend between these groups, with collagen-related genes increasing over time and soft ECM-related genes decreasing. Although the conduction-related gene patterns did not perfectly align with ECM gene trends, the findings provide intriguing insights and point to the potential for new hypotheses regarding the interaction between ECM and conduction system development.

2. METHODS

2.1 Data Source and Processing

The single-cell RNAseq data used in this study was obtained from GSE149457. H&E-stained tissue images and spatial metadata were obtained from the GitHub repository (https://github.com/madhavmantri/chicken_heart) . The downloaded data was processed and annotated by the data provider.

Table 1. The total number of cells for each cell type

Heart stages	Regions	# cells
D4	Atria	70
	Epicardium-like	184
	Outflow tract	154
	Valve	113
	Ventricle	226
D7	Atria	340
	Compact LV and inter-ventricular septum	393
	Endothelium	314
	Epicardium	216
	Right Ventricle	191
	Trabecular LV and endocardium	367
	Valve	145

Heart stages	Regions	# cells
D10	Atria	327
	Compact LV and inter-ventricular septum	502
	Epicardium	240
	Outflow tract	55
	Right Ventricle	271
	Trabecular LV and endocardium	274
	Valve	247
D14	Atria	454
	Compact LV and inter-ventricular septum	693
	Epicardium	130
	Right Ventricle	286
	Trabecular LV and endocardium	188
	Valve	216

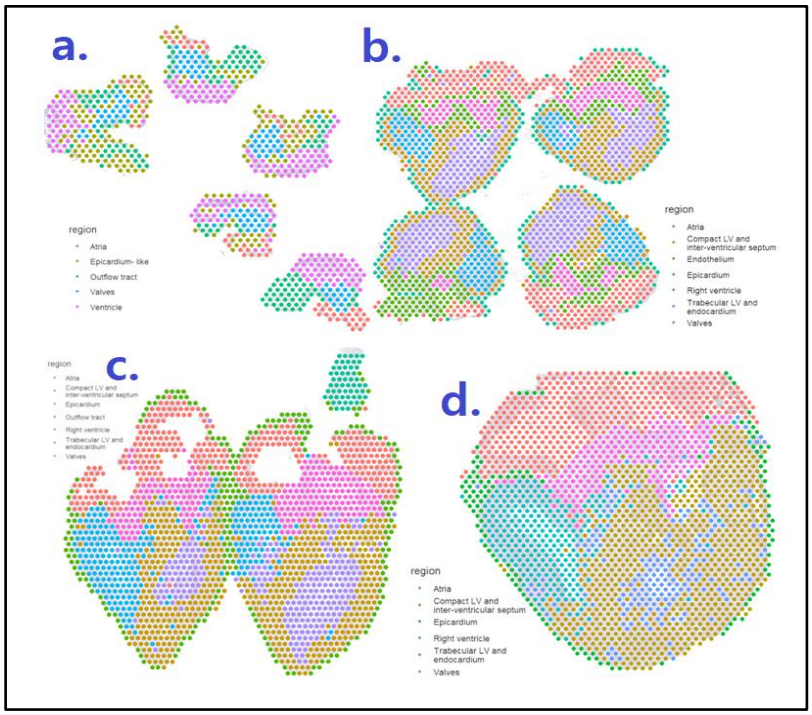

Fig. 1. Region annotations used for spatial transcriptomics analysis. a. Day 4 heart has atria, epicardium-like, outflow tract, valves, and ventricle regions. b. Day 7 heart has atria, compact LV and inter-ventricular septum, endothelium, epicardium, right ventricle, trabecular LV and endocardium and valves regions. c. Day 10 heart has atria, compact LV and inter-ventricular septum, epicardium, outflow tract, right ventricle, trabecular LV, and endocardium and valves regions. d. Day 14 heart has atria, compact LV and inter-ventricular septum, epicardium, right ventricle, trabecular LV, and endocardium and valves regions

2.2 Region-specific Spatial Transcriptomics Analysis

Each cell row in the count matrix corresponds to spatial location metadata linked to H&E images. Using the metadata provided in the GitHub repository, we reconstructed the spatial mapping of gene expression. Fig. 1 illustrates the region annotations used for the analysis. Based on these regions, the expression levels of various genes were plotted to quantitatively highlight region-specific patterns. We manually reviewed ECM-related genes, ion channels (sodium, potassium, etc.), and cardiac conduction-related genes to select 12 genes (COL1A1, COL3A1, DCN, MMP2, HAPLN1, FBLN1, BMP4, ITGA8, GJA1, GJA5, TBX5, and CACNA1D) that exhibited clear trends across the developmental stages.

3. RESULT

3.1 Overview of ECM-related Gene Expressions in Various Regions and Development Stages

Figs. 2 and 3 below display the overall expression coverage (the percentage of cells expressing at least one transcript of a gene), represented by the size of the dots, and the normalized expression levels, indicated by the color of the dots. The first eight genes shown are ECM-related (COL1A1, COL3A1, DCN, LAMB2, MMP2, HAPLN1, FBLN1, and ITGA8), while the last four genes are conduction-related (GJA1, TBX5, CACNA1D, and GJA5). These genes were selected based on their differential expression across the developmental stages.

Collagen-related genes (COL1A1, COL3A1, and DCN) show high expression levels in the later stages, with a clear correlation between expression and developmental progression. In contrast, genes related to the softer components of the ECM (HAPLN1 and MMP2) are more highly expressed in the earlier stages, with their expression levels decreasing as development progresses. The conduction-related genes exhibit more complex patterns. GJA5 peaks at day 7 but declines in the later stages, while GJA1 is predominantly expressed in the day 4 heart. TBX5, a transcription factor involved in the development of the conduction system, follows a similar trend to GJA5, peaking at day 7 and decreasing thereafter. The calcium channel gene, CACNA1D, is more abundantly expressed in the earlier stages and decreases as the heart matures.

The spatial regions of expression are summarized in Fig. 3. Collagen-related genes are primarily expressed in the epicardium and valves, while genes associated with softer ECM components are found in the outflow tract (HAPLN1) or in the valves and ventricles (MMP2). Given that HAPLN1 and MMP2 are predominantly expressed in the earlier developmental stage (day 4), their expression is localized to precursor regions (outflow tract) or regions that overlap with other developmental structures (valves and ventricles). Fibulin 1 (FBLN1) and bone morphogenetic protein 4 (BMP4) were found to be specifically expressed in the epicardium, with expression peaking at day 10 and being highly localized. Although ITGA8 did not show a distinctive pattern in the global analysis, more specific spatial analysis revealed very localized expression of the gene in the valve.

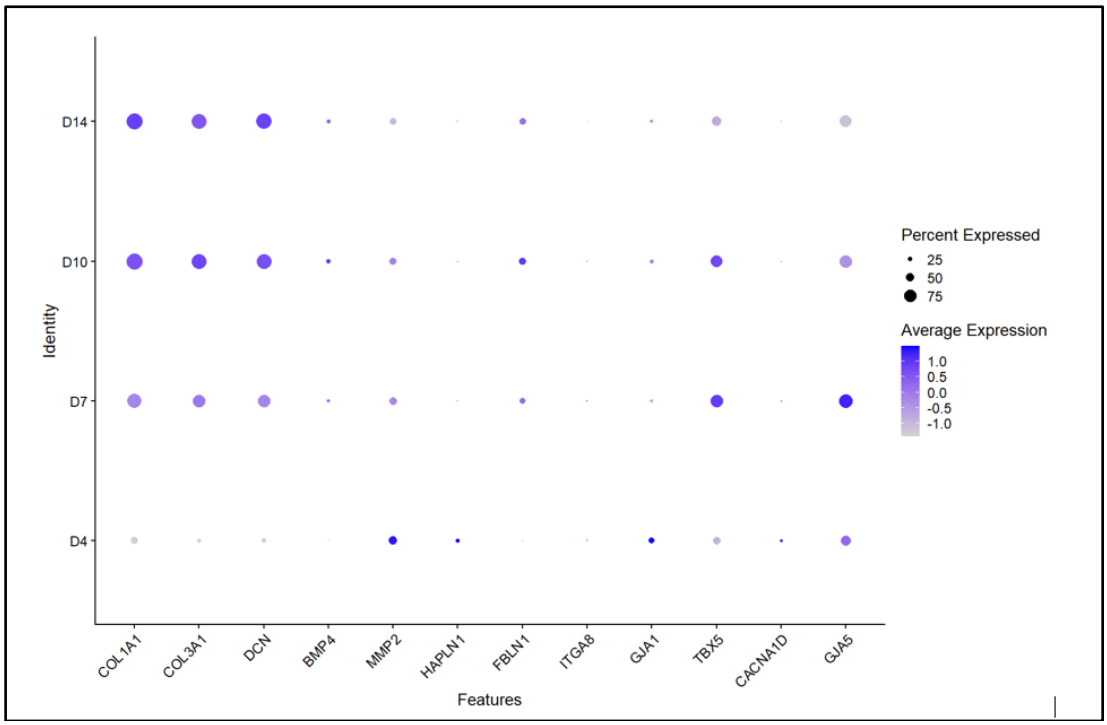

Fig. 2. Gene expression levels and expression coverage for different development stages

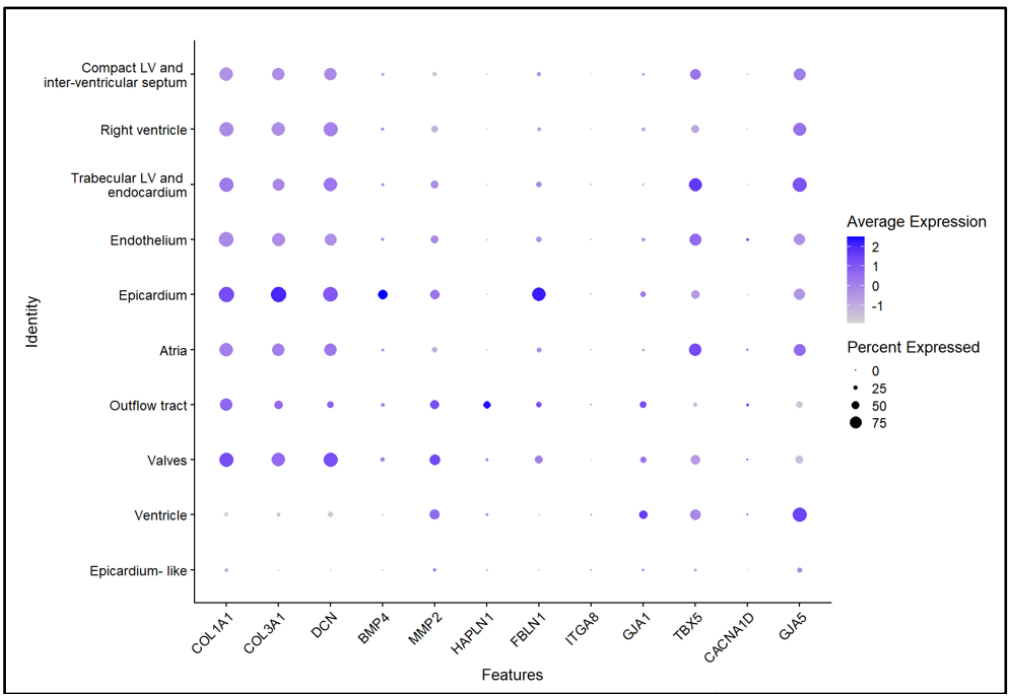

Fig. 3. Gene expression levels and expression coverage for different spatial regions

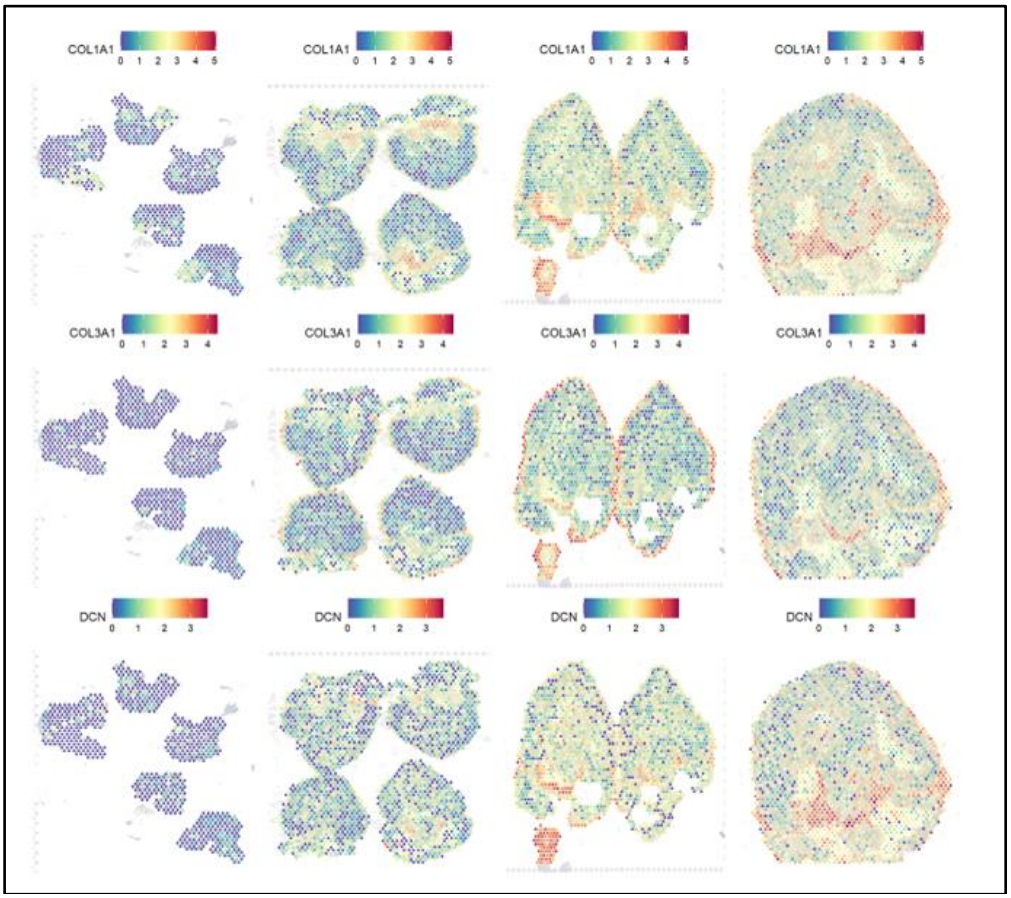

Fig. 4. The spatial expression pattern of collagen 1A1 (COL1A1), collagen 3A1 (COL3A1) and decorin (DCN) are depicted for D4, D7, D10 and D14 hearts

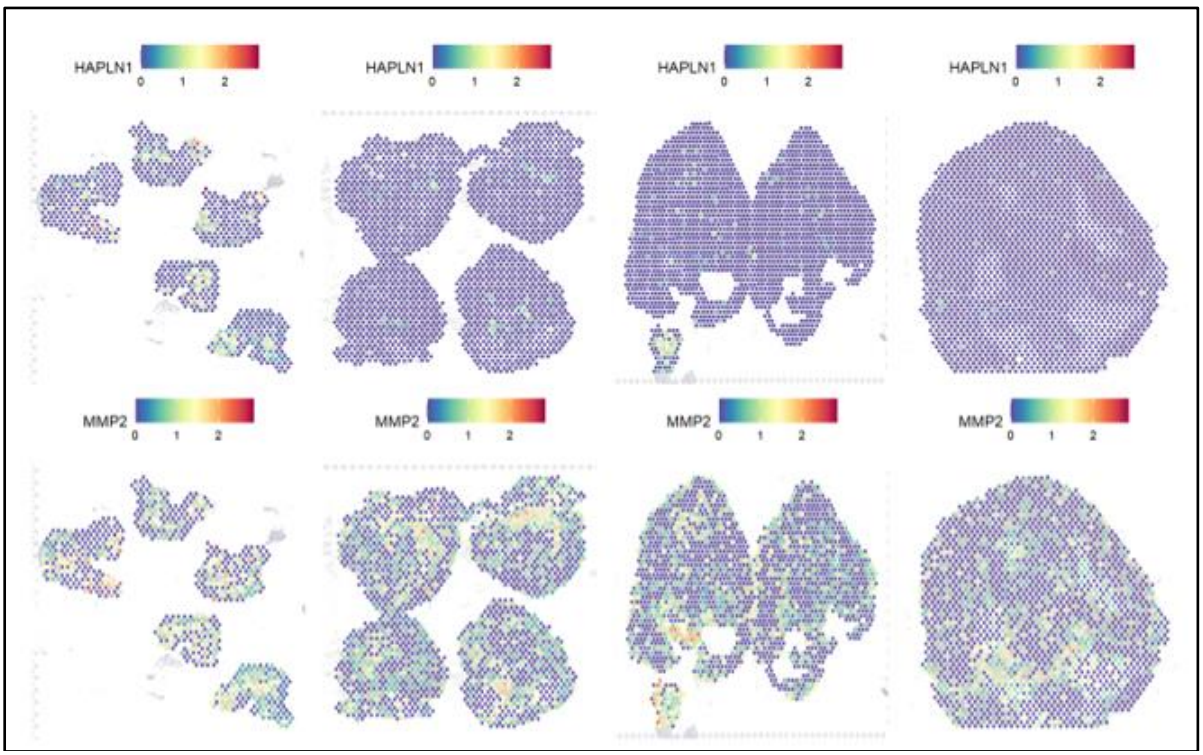

Fig. 5. The spatial expression pattern of hyaluronan and proteoglycan link protein 1 (HAPLN1) and matrix metalloproteinase 2 (MMP2) are depicted for D4, D7, D10 and D14 hearts

3.2 Collagen and Collagen-Related Genes Increased in Expression as the Heart Develops

In Fig. 4, selected collagen and collagen-related genes were mapped to spatial coordinates to generate a heart map. Decorin (DCN), a molecule known to bind with collagen to form collagen fibrils, exhibits spatial expression closely correlated with collagen genes. Starting from day 7, there is a strong expression of COL1A1 and COL3A1 in the valves and epicardium. DCN also shows elevated expression in these regions. This pattern continues through day 14, by which time the heart has matured into a fully functioning organ with four chambers and valves.

3.3 Molecules Related to Soft ECM Composition Decreased in Expression as the Heart Develops

In contrast to collagen-related genes, MMP2 and HAPLN1 are highly expressed in the earlier stages of heart development, with their expression decreasing in later stages. Notably, HAPLN1 expression is predominantly found in day 4 hearts and is localized to the outflow tract by day 10 as in Fig. 5. Since the outflow tract represents an immature part of the heart that diminishes as the heart matures, HAPLN1 serves as a valuable marker for early heart development. MMP2, on the other hand, is expressed at all stages, but from day 7 onward, its expression becomes primarily localized to the valves.

3.4 Cardiac Action Potential Conduction-Related Genes

GJA1 and GJA5 are integral ion channels that facilitate the free flow of ions between cardiac cells, thereby increasing the conduction velocity of the action potential wave originating from the sinus node. Both GJA1 and GJA5 show high protein expression in the ventricles as in Fig. 6. GJA1 is highly expressed at day 4, but its expression decreases in the later stages of development. In contrast, GJA5 expression increases until day 7 and then declines. TBX5, a key gene involved in the development of the cardiac conduction system and Purkinje fibers, shows localized expression in the trabecular region of the left ventricle.

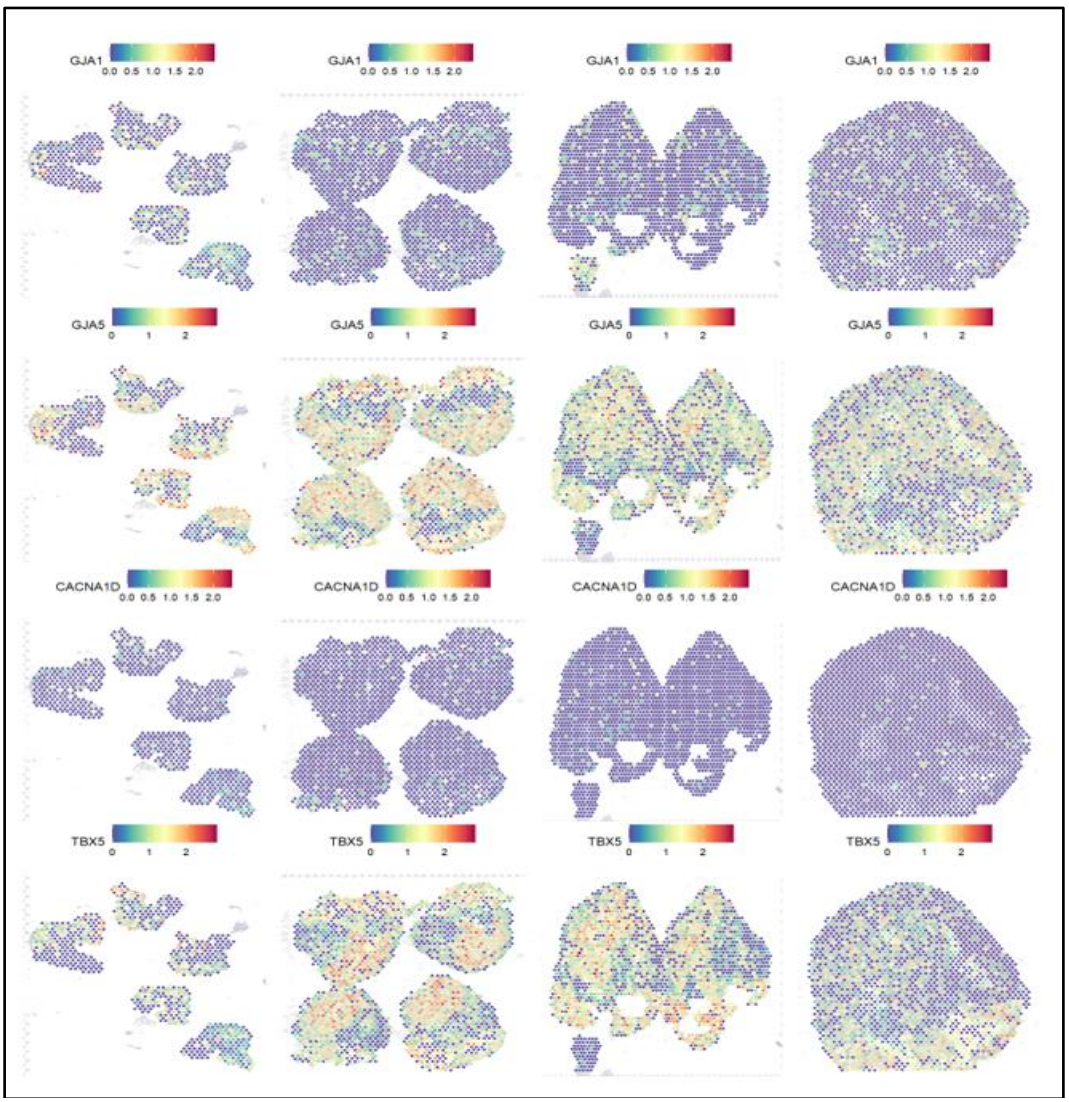

Fig. 6. The spatial expression pattern of gap junction protein alpha 1 (GJA1), gap junction protein alpha 5 (GJA5), calcium channel, voltage-dependent, L type, alpha 1D subunit (CACNA1D), and T-box 5 (TBX5) are depicted for D4, D7, D10 and D14 hearts

3.5 Fibulin 1 (FBLN1), Bone Morphogenetic Protein 4 (BMP4), and ITGA11 Highly Expressed in Epicardium and Valves

We examined other genes with localized expression patterns across developmental stages. FBLN1 and BMP4 are both highly expressed in the epicardium but not in other regions, suggesting their roles are specifically related to maintaining the structural integrity of the heart as in Fig. 7. ITGA11 is uniquely expressed in the valves, indicating a region-specific function. These genes appear to be activated around day 7 and remain upregulated thereafter.

4. DISCUSSION

4.1 Change in Mechanical Properties of ECM in Cardiac Tissue

As demonstrated in Figs. 2 and 4, there is a clear upward trend in the expression levels of collagen and collagen-related genes as the heart develops. The increase in collagen is observed not only in supporting tissues, such as the epicardium, but also in functional tissues like the myocardium. This distinguishes collagen-related genes from others, such as FBLN1, BMP4, and ITGA11, which are localized to connective tissues. These findings suggest that collagen plays a crucial role in regulating the mechanical

properties of the entire heart. The expression of collagen-related genes notably increases around day 7, becoming ubiquitous across all heart regions. In contrast, at day 4, collagen expression is largely restricted to the epicardial regions.

On the other hand, the expression of HAPLN1 and MMP2 decreases by day 7. Since these two genes are associated with the production of softer ECM materials and the breakdown of collagen, it is reasonable to infer that the heart at day 4 is considerably softer than in later stages. This suggests a major remodeling of cardiac tissue and ECM occurs between days 4 and 7.

4.2 Mechanical Properties of ECM and Cardiac Conduction

Our hypothesis that ECM's mechanical properties influence cardiac conduction cannot be fully determined with the available data. In Fig. 6, GJA1 shows a clear trend, with its expression level declining significantly after day 4, which appears to coincide with ECM remodeling. However, GJA5 and TBX3 present mixed results, with their expression levels increasing up to day 7 before declining thereafter. This may indicate that the cells produced a sufficient amount of the corresponding proteins and shifted into a maintenance mode, where no additional expression of these molecules was required. These findings suggest that ECM remodeling and the construction of the cardiac conduction system likely occurred between day 4 and day 7. The rebuilding of the tissue appears to continue until around day 10, after which the majority of the cardiac infrastructure is already established, leading to a decline in further expression.

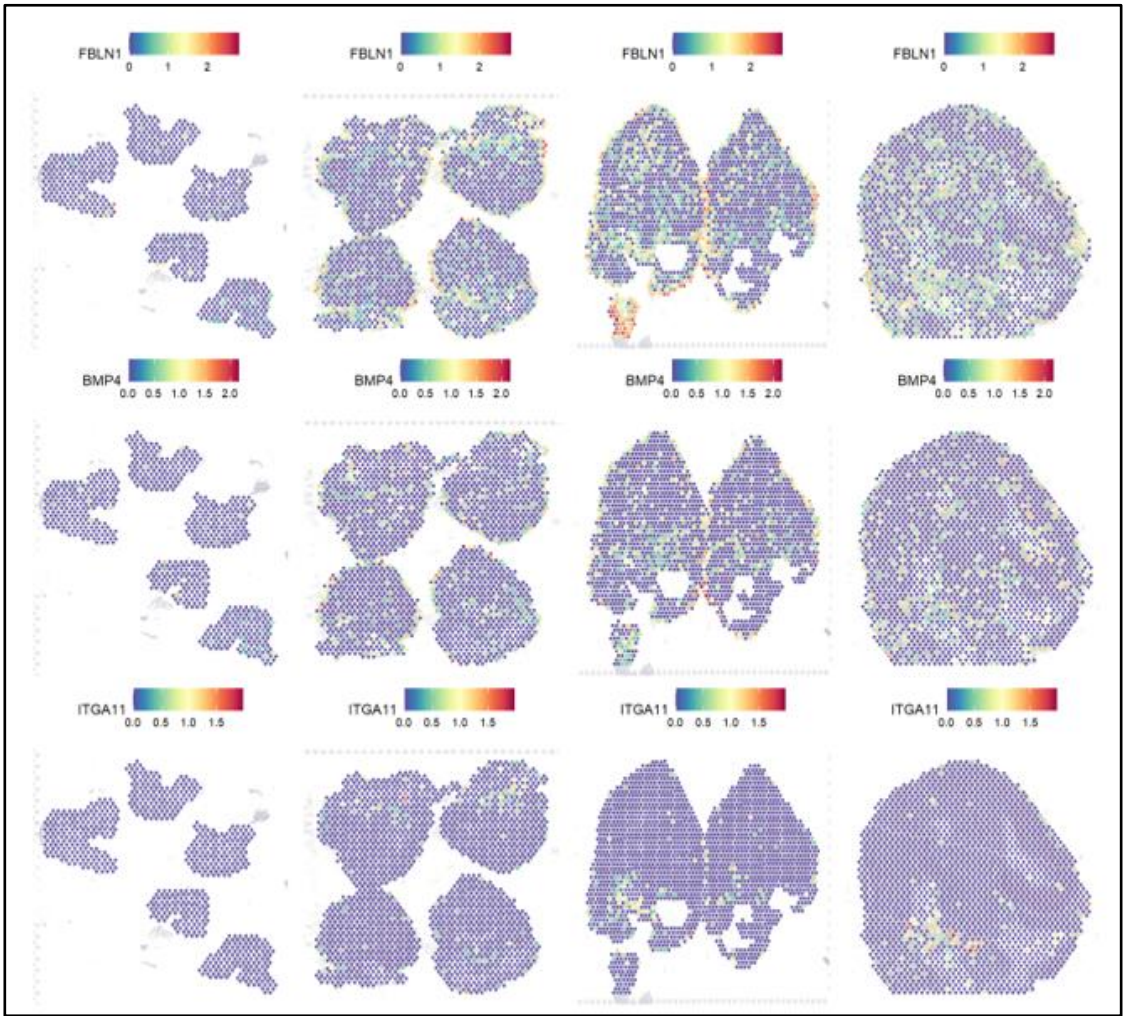

Fig. 7. The spatial expression pattern of Fibulin 1 (FBLN1), bone morphogenetic protein 4 (BMP4) and integrin subunit alpha 11 (ITGA11) are depicted for D4, D7, D10 and D14 hearts

4.3 Limitations of the Study

There are limitations in the study that need to be addressed to further validate and support the hypothesis. The data contained a limited number of cells per region (see Table 1), which reduced the number of genes that could be reliably analyzed. Other key genes, such as HCN family or SCN family genes, were screened out because of very low expression coverage (less than 10% of total cells expressing any levels of the genes). More genes can be added to this study if there were more cells available for analysis. Furthermore, any molecular pathway implication made with the study needs to be supported with in-vivo/in-vitro experiments with proper controls and experimental variables.

5. CONCLUSION

The study highlights the dynamic changes in ECM composition during heart development, particularly the increasing expression of collagen-related genes and the concurrent decrease in soft ECM components. These changes suggest that ECM remodeling plays a key role in the maturation of cardiac tissues, including the conduction system. While the exact influence of ECM on cardiac conduction remains unclear, the data point to a critical period between days 4 and 7 where significant remodeling occurs. Further in vivo and in vitro studies are necessary to fully understand the relationship between ECM mechanical properties and the development of the cardiac conduction system. This study lays the groundwork for future research into how the ECM contributes to the mechanical and electrical functionality of the heart during development.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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