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Review

Genetics and Molecular Basis of Congenital Heart Defects in Down Syndrome: Role of Extracellular Matrix Regulation

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Abstract: Down syndrome (DS), a complex disorder that is caused by the trisomy of chromosome 21 (Hsa21), is a major cause of congenital heart defects (CHD). Interestingly, only about 50% of individuals with Hsa21 trisomy manifest CHD. Here we review the genetic basis of CHD in DS, focusing on genes that regulate extracellular matrix (ECM) organization. The overexpression of Hsa21 genes likely underlies the molecular mechanisms that contribute to CHD, even though the genes responsible for CHD could only be located in a critical region of Hsa21. A role in causing CHD has been attributed not only to protein-coding Hsa21 genes, but also to genes on other chromosomes, as well as miRNAs and lncRNAs. It is likely that the contribution of more than one gene is required, and that the overexpression of Hsa21 genes acts in combination with other genetic events, such as specific mutations or polymorphisms, amplifying their effect. Moreover, a key function in determining alterations in cardiac morphogenesis might be played by ECM. A large number of genes encoding ECM proteins are overexpressed in trisomic human fetal hearts, and many of them appear to be under the control of a Hsa21 gene, the RUNX1 transcription factor.

Keywords: Down syndrome; chromosome 21; congenital heart defects; extracellular matrix; *RUNX1*

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1. Introduction

Congenital heart defects (CHDs) refer to a range of heart developmental anomalies occurring in ≈1% of live births [1,2]. CHDs are present with approximately a 50% recurrence in Down syndrome (DS) subjects [3,4]. The most frequent anomalies in DS are endocardial cushion defects [5] such as atrioventricular canal defects (AVCD), ventricular septal defects (VSD), atrial septal defects (ASD), patent ductus arteriosus (PDA) and tetralogy of Fallot (ToF) [6,7]. Since CHD is a very frequent sign in DS, it seems plausible that an altered expression of gene(s) mapping to chromosome 21 (Hsa21) might play a role in cardiac development.

Some years ago, independent studies suggested that most signs of the DS phenotype were associated with three copies of chromosome band 21q22.2–22.3, which was named the Down syndrome critical region (DSCR) [8–10]. Later, a critical region responsible for the cardiac phenotype in DS patients, the DS-CHD, was established in a 9 Mb Hsa21 region including *D21S55* through the telomere [11]. This region was then narrowed down to approximately a 4 Mb region, from *D21S55* to *MX1* [12,13]. In 2001, Barlow et al. proposed a new candidate DS-CHD region spanning from *D21S3* to *PFKL* [14]. This segment was further limited to a region spreading less than 2 Mb from *DSCAM* to *ZNF295* [15] (Figure 1). Recently, a more restricted region spanning about 1 Mb and containing three protein-coding genes (*DSCAM*, *BACE1* and *PLAC4*), was proposed [16].

Most of these studies, based on human partial trisomies, are hampered by the fact that the karyotype of the examined individuals was complex, including other chromosome

anomalies, and the phenotype was quite heterogeneous and rare. For this reason, mouse strains have been generated to recapitulate the pathology and genetics of DS-CHD [17,18]. In the DS mouse model Dp(16), Liu et al. identified a genomic region associated with CHD similar to that observed in DS subjects [19]. This region, which spans from *Tiam1* to *Kcnj6* and includes 52 Hsa21 ortholog genes, was further narrowed to 3.7 Mb from *Ifnar1* to *Kcnj6* (35 Hsa21 ortholog genes) [20]. Lana-Elola et al., using a mapping panel of seven mouse strains, proposed as DS-CHD a genomic region from *Mir802* to *Zbtb21*, partially overlapping what was previously described by Liu [21] (Figure 1).

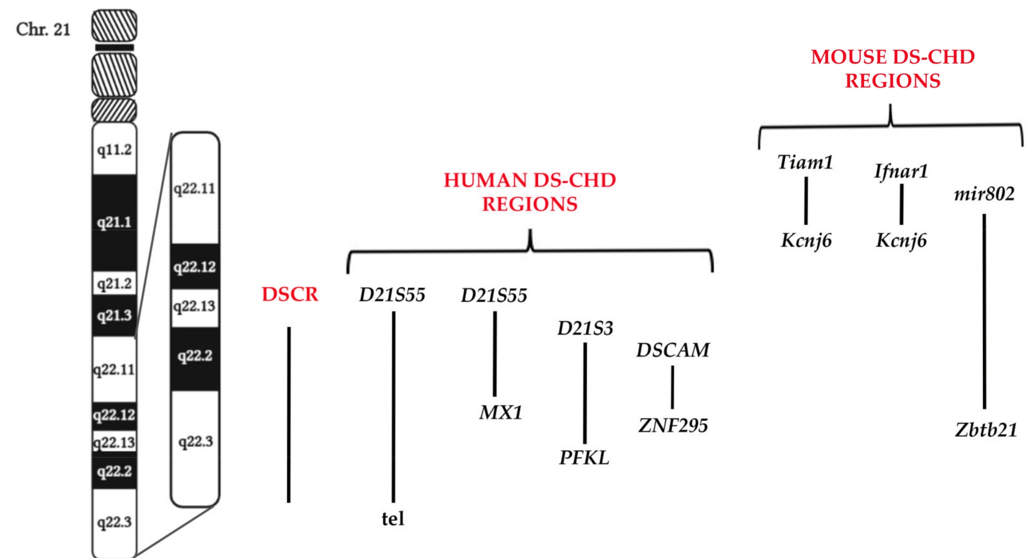

Figure 1. Schematic representation of DS phenotype critical regions. The 'Down Syndrome Critical Region' (DSCR) [8–10] and human [11–15] and mouse [19–21] DS–CHD regions with corresponding boundaries have been defined using the GRCh38/hg38 UCSC assembly.

In any case, whatever the critical region for CHD in DS, multiple findings suggest that cardiac defects in DS are not the result of the overexpression of a single gene, but of a set of them [22].

In this review, we address the role of genes mapping to Hsa21 and other chromosomes in the development of the heart and in molecular mechanisms responsible for CHD. Since the extracellular matrix (ECM) is known to play a crucial role in the morphogenesis of the heart, we focus on those genes that code for proteins involved in ECM assembly and function, and whose expression is dysregulated as a consequence of Hsa21 trisomy.

2. Candidate Genes for CHD in DS

2.1. Genes Mapping to Hsa21

As trisomy 21 (T21) is the genetic abnormality most commonly associated with heart defects, studying the contribution of Hsa21 genes in the development of the heart could be an entry point for better understanding the pathogenesis of CHD.

Many experiments have been carried out to understand the molecular mechanisms leading to CHD in DS. Several genes mapping to Hsa21 are involved in embryonic cardiogenesis. Moreover, it has been proposed that altered Hsa21 gene dosage can magnify the effects of either pathogenic mutations or polymorphisms in other genes [6]. The transcriptional profile of human hearts from DS fetuses demonstrated that most Hsa21 genes are overexpressed in the developing heart due to gene dosage [23].

Some evidence suggests the existence of genes on Hsa21, involved in cell adhesion, which likely play an important role in valvulo-septal morphogenesis. If overexpressed, these genes may cause cardiac defects [14,24,25]. One of these genes is *DSCAM*, a member of the immunoglobulin superfamily of cell adhesion molecules (Ig-CAMs) [26]. It is expressed

during cardiac development before endocardial cushion fusion. Its overexpression, due to the trisomy, is responsible for increased cell–cell adhesion [14,27]. This may induce alterations in the cushion development [14].

In a similar way, the overexpression of collagen VI, two of three chains of which map to Hsa21, has been associated to cardiac septal defects in DS [28,29]. Fetal human heart staining demonstrated that collagen VI is present in the atrioventricular (AV) cushions and that its expression is higher in T21, possibly altering their development [29]. Collagen VI is an activator of discoidin domain receptors (DDRs) [30], which regulate cell–collagen interactions and, in turn, induce the expression of metalloproteases and other ECM proteins [31]. DDR2 expression has been detected by confocal microscopy in the developing heart, within the cardiac cushions and eventually within the septum [32]. *DDR1* and *DDR2* are upregulated in trisomic heart samples [23].

Interestingly, septal defects have been observed when both *DSCAM* and *COL6A1* are co-expressed in the murine heart, indicating that the overexpression of these two genes may exert synergistic effects on cardiac defects [33], even though Kosaki et al. argued that triplication of the *DSCAM* gene alone might be the cause of cardiac defects in DS [34].

Two other Hsa21 genes are potential contributors to AVSD in DS patients: the dual-specificity tyrosine phosphorylation-regulated kinase 1A (*DYRK1A*), and the regulator of calcineurin 1 (*RCAN1*), also known as *DSCR1*. Synergistically, both cause a decrease in the activity and levels of the transcription factors belonging to the nuclear factor of active T cell (NFATc) family [35].

DYRK1A encodes a nuclear serine/threonine kinase that primes substrates for phosphorylation by GSK3, which phosphorylates NFATc proteins in the nucleus, resulting in their inactivation and export [36].

RCAN1 encodes a binding protein that negatively controls calcineurin activity [37]. Calcineurin is a unique serine/threonine protein phosphatase under the control of Ca^{2+}/calmodulin: an increase in intracellular Ca^{2+} leads to the activation of calcineurin [38], which then dephosphorylates NFATc proteins, causing their nuclear entry and assembly with partner proteins (NFATn) to form NFAT transcription complexes [39]. The NFAT signaling pathway, a critical regulator of vertebrate development and organogenesis [40], is important for the cardiac valve and septum morphogenesis [41–43]. NFATc mutant mice manifest vascular and cardiac morphogenic defects [36]. Striking similarities have been detected between the phenotypic features of DS and mice carrying deletions of genes encoding components of the NFAT signaling pathway, and 65% of NFATc1–4-null mice have endocardial cushion defects [35].

Acting as a negative regulator of the NFAT signaling pathway, the overexpression of *DYRK1A* and *RCAN1* genes in DS may be considered a putative cause of cardiac development defects in DS patients [44,45]. *NFATc* genes were found to be downregulated in the heart tissues of fetuses with DS, while *DYRK1A* was upregulated [23]. *RCAN1* expression was greatly variable and not overall significantly dysregulated in DS fetal hearts, but its expression was inversely correlated with that of *NFATc3* [23].

Interestingly, DYRK1A and RCAN1 are also crucial regulators of Synaptojanin 1 (*SYNJ1*), another Hsa21 gene encoding a polyphosphoinositide phosphatase. The former, phosphorylating SYNJ1, the latter implicated in its dephosphorylation, finely modulate the SYNJ1 activity [46,47].

SYNJ1 is a key player of early endosomal compartments, regulating their homeostasis and functions in different cell types [48–50]; on the other hand, a dangerous liaison between Synj1 and endosomal trafficking has been observed in DS [51,52]. Compelling evidence has shown that endosomal transport is critical for heart functions [53], controlling the localization and turnover of cardiac proteins, such as cardiac pacemaker channels [54] and sodium channels [55], suggesting a potential role of this pathway in the cardiac defects of DS.

The notion that only half of DS subjects present CHD suggests that some other events, such as copy number variations (CNV), SNPs, or other genetic anomalies, may concur with trisomy to alter heart development [56].

CNVs of Hsa21 tracts have been associated with AVSD risk in DS. The analysis of CNV in DS subjects with AVSD revealed three regions associated with an AVSD risk. Two of them are located within the previously identified DS-CHD region on Hsa21: the former maps near the *RIPK4* gene, and the latter in the *ZBTB21* (previously *ZNF295*) gene, suggesting the potential role of these genes in the pathogenesis of CHD in DS [56]. This last gene was overexpressed in the hearts of fetuses with CHD when compared with DS hearts without CHD (dataset from ref. [21], unpublished results). The *ZBTB21* gene has also been shown to interact with *PPP2R2B*, which regulates the WNT/β-catenin signaling pathway in drosophila. This pathway is required for cardiac differentiation in human embryonic stem cells [57].

Finally, relationships have been established between SNPs in two interferon receptor genes (*IFNAR1* and *IL10RB*) and CHD in subjects with DS [58]. These receptors map to the 3.7 Mb critical genomic region associated with DS heart defects described by Liu et al. [20].

2.2. Genes Mapping to Other Chromosomes

The role of non Hsa21 genes in causing CHD has been demonstrated in humans and experimental mice by studies not related to DS.

Rare variants in *BMP4*, *CRELD1*, *CRELD2*, *FBLN2*, *FRZB*, *GATA4* and *GATA5* genes have been found in individuals with DS and complete AVSD, suggesting that rare genetic variants in these genes, incompletely penetrant on an euploid background, may act synergistically with T21 to increase the risk for AVSD in DS [59–62]. *CRELD1* (mapping to 3p25) encodes a cell surface protein that might participate in cell adhesion. Maslen et al. found two *CRELD1* missense mutations in children with DS and AVSD, implying its role in the pathogenesis of the disease [5]. A pathway analysis proved that all these genes, together with the DDRs cited above, are somehow associated with the vascular endothelial growth factor-A (VEGF-A) pathway [60,63–66].

Several studies highlight the potential contribution of the VEGF-A and calcineurin/NFAT pathways to CHD development in DS patients [37,60]. VEGF-A is a strong mitogen known as a regulator of AV valvuloseptal morphogenesis, wherein it is critical for the formation of the AV endocardial cushions and helps the morphogenesis of those primordial structures into the AV valves [67,68]. Studies in animal models have shown that altered expression levels of VEGF-A during heart development are associated with CHD, including AVSD [69–72], and that *VEGF-A* overexpression in mouse embryos triggers severe heart developmental abnormalities [73].

An imbalance of overexpressed Hsa21 genes is known to affect the expression of genes located in different chromosomes [23,74,75]. More than 400 genes that do not map to Hsa21 were found to be dysregulated in the heart tissues of fetuses with DS [23]. It was observed that the most dysregulated category consisted of genes coding for mitochondrial proteins belonging to all five complexes, as well as proteins involved in mitochondrial biogenesis and function. The fact that these nuclear-encoded mitochondrial genes (NEMGs) were all downregulated suggested that the corresponding proteins and enzymatic activities might be reduced in DS cells, and that mitochondrial function could be consequently impaired [76]. It was indeed determined that the mitochondria of DS fibroblasts presented an altered ultrastructure, with breaks in the inner membrane and alterations in the mitochondrial cristae, a reduced oxygen consumption rate (OCR) and mitochondrial membrane potential, and an increase in intracellular ROS production [76]. These findings are relevant in light of the fact that alterations of mitochondrial morphology have been shown to affect cardiomyocyte differentiation, apoptosis and autophagy [77], and that mitochondrial activity and energy conversion play a central role in the normal function of the heart. Mitochondrial dysfunction is considered to be one of the relevant mechanisms that plays a role in the pathogenesis of cardiovascular diseases [22,78]. Furthermore, the

comparison between fibroblasts from DS fetuses with and without heart defects led to the intriguing observation that mitochondrial dysfunction was more severe in fibroblasts from cardiopathic trisomic fetuses, which presented a more pronounced pro-oxidative state [76], suggesting that mitochondria may be an interesting target in the pathogenesis of CHD. It is worth mentioning that the NEMGs downregulated in T21 fetal hearts were under the control of *PGC-1α*, which is, in turn, regulated by the Hsa21 gene *NRIP1*. The attenuation of *NRIP1* expression by siRNAs in trisomic fibroblast [79] or the promotion of *PGC-1α* activity via metformin [80] or pioglitazone [81] were able to restore the level of expression of NEMGs and to counteract mitochondrial dysfunction [82,83]. It will be of interest to further explore the role of *NRIP1*, *PGC-1α*, NEMGs and mitochondrial dysfunction in the pathogenesis of CHD.

Two of the genes that were found to be downregulated in the heart tissues of fetuses with T21 [23], namely *TBX20* and *SRF*, deserve particular attention.

The cardiac T-box factor *Tbx20* is expressed in the early cardiac progenitor region, endocardium and myocardium, endothelial cells of the outflow tract (OFT), endocardial cushions and AV cushions, the precursor structure of cardiac valves and the atrioventricular septum. It directly interacts with Nkx2-5, GATA4 and GATA5 in regulating gene expression in the developing heart [84] and acts as a key upstream regulator for a variety of other genes, such as *Bmp2*, *Tbx3* and *Hand1*, in the early atrio-ventricular channel (AVC) development [85]. *Tbx20* is, therefore, essential for AVC patterning and cushion formation, playing crucial roles in these two important aspects to coordinate early heart development [85]. Mutations in *TBX20* are widely associated with the complex spectrum of CHD in humans, which includes defects in chamber septation, chamber growth and valvulogenesis [84].

Serum response factor (SRF) is a major transcription factor that controls both embryonic and adult cardiac development. *SRF* expression is needed throughout development, from the first mesodermal cell in a developing embryo to the last cell damaged by infarction in the myocardium. *Srf* indirectly regulates *Hand2* expression through a miRNA-mediated mechanism [86]. The precise regulation of *SRF* expression is critical for mesoderm formation and cardiac crescent formation in the embryo, and altered *SRF* levels lead to cardiomyopathies in the adult heart, suggesting its vital role in cardiac development and disease [87].

2.3. Role of ncRNAs in the Pathophysiology of CHD in DS

Several microRNAs mapping to Hsa21 are overexpressed in DS [88,89]. *MiR-99a*, *let-7c*, *miR-125b-2*, *miR-155* and *miR-802* were found to be overexpressed in the cardiac tissue of patients with T21 [90,91]. It has been demonstrated that cardiomyogenesis is controlled by the *miR-99a/let-7c* cluster [88]. When overexpressed, *let-7c* promotes cardiomyogenesis by upregulating the genes involved in mesoderm specification (*T/Bra* and *Nodal*) and cardiac differentiation (*Mesp1*, *Nkx2.5* and *Tbx5*), repressing its direct target *EZH2*. On the contrary, *miR-99a* represses cardiac differentiation, targeting *Smarca5* in mouse. Indeed, *EZH2* and *SMARCA5* were found to be downregulated in DS fetal hearts, suggesting that they might participate in CHD pathogenesis [88].

Another let-7c target, *SLC25A4/ANT1*, was identified and validated by Izzo et al. [91]. This gene, which is downregulated in DS fetal hearts [23], is the main translocator of ADP/ATP across the mitochondrial membrane; therefore, its repression might have a potential negative impact on mitochondrial function. It is known that mitochondrial dysfunction might affect several DS features, including cardiac alterations [22,92].

It was reported that Hsa21 miR-155-5p affects mitochondrial biogenesis by targeting the mitochondrial transcription factor A (*TFAM*), a gene that was found to be downregulated in trisomic hearts [93]. TFAM is a nuclear-encoded protein that controls the transcription and maintenance of mtDNA and, therefore, mitochondrial biogenesis.

Among miRNAs that do not map to Hsa21, *miR-1* has been shown to regulate cardiac differentiation and control heart development in mice determining miRNA-guided translational inhibition and repression of the cardiac transcription factor Hand2 [94]. Particularly,

miR-1 is regulated by the transcription factor SRF [95], which is downregulated in DS fetal hearts [23]. Its expression is consequently downregulated, causing an excessive synthesis of *HAND2* (unpublished data), which is a possible cause of VSD.

Recent investigations have focused on the role of lncRNAs in the pathophysiology of CHD in DS. For instance, a genome-wide association study in DS subjects identifies in the FLJ33360 lncRNA a common SNP variant associated with an increased risk of DS-associated AVSD. FLJ33360 maps to chromosome 7. Interestingly, its adjacent gene *MED10* has been associated with cardiac defects [96] and participates in heart valve formation in zebrafish by affecting the expression of the transcriptional regulator Tbx2b in the AV myocardium [97].

3. Role of ECM Regulation in the Development of CHD in DS

The early stages of development of the cardiovascular system, as for other body systems, are characterized by a primordial ECM, whereas later developmental stages are characterized by resorption of this matrix and transition to a mature collagen- or elastin-rich ECM, a process that continues into the neonatal period [98]. The cardiac ECM is a dynamic, robust, and functionally versatile component that forms the non-cellular part of the cardiac muscle. It acts not only as an architectural scaffold that supports the cardiac cells, but also actively participates in the development and differentiation of cardiac and vascular cells [99]. Indeed, the cellular response to changes in ECM can trigger epithelial-mesenchymal transformation (EMT), the developmental process thought to be responsible for heart valve and septa development [100].

Endocardial cushions consist of an ECM jelly, containing highly proliferative valve progenitor cells and susceptible to the continual remodeling of its components, which is necessary for subsequent morphogenetic events [101]. The presence of ECM jelly is fundamental in the ventricular wall and endocardial cushions during their formation.

The transcriptional profile of human hearts from DS fetuses demonstrated that genes coding for ECM proteins, proteoglycans, collagens and multi-adhesive matrix proteins are over-represented among the upregulated ones [23]. A meta-analysis of 45 heterogeneous DS data sets [102] confirmed this trend, as a functional analysis of the 324 genes consistently upregulated in trisomic samples indicated that 37 of them belonged to the ECM Cell Component GO category. A significant dysregulation of genes coding for ECM components has also been reported in other meta-analysis studies [103,104].

Given this fundamental role of ECM in heart development, it is conceivable that the consequence of gene dysregulation on ECM composition and assembly may contribute to congenital anomalies, including cardiac defects [4,29].

More than 40 genes encoding ECM proteins were found to be upregulated in DS fetal heart tissues [23]. This group included genes mapping to Hsa21, such as members of the ADAMTS protein family (*ADAMTS1* and *ADAMTS5*), *APP*, *JAM2* and collagens (*COL6A1*, *COL6A2* and *COL18A1*), which are dose-dependently upregulated in trisomic samples. It also includes genes that do not map to Hsa21, such as collagen type I (*COL1A1* and *COL1A2*), type III (*COL3A1*), type V (*COL5A1* and *COL5A2*), type 9 (*COL9A2* and *COL9A3*), type 13 (*COL13A1*), type 14 (*COL14A1*), type XV (*COL15A1*), versican (*VCAN*), fibronectin (*FN1*), fibulin (*FBLN1*), metalloproteases (*MMP2* and *MMP11*) and several adhesion molecule genes, which are likely regulated by the overexpression of Hsa21 genes. All these genes are supposed to affect cell adhesion properties, possibly determining an increase in adhesiveness [23]. Their function and potential role in CHD are listed in Table 1.

Table 1. ECM-related genes dysregulated in DS, possibly involved in the occurrence of CHD.

Gene Name	Location	Gene Product	Potential Role in CHD
ADAMTS1 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 1)	21q21.3	Cleaves aggrecan, a cartilage proteoglycan, at the '1938-Glu-|-Leu-1939' site (within the chondroitin sulfate attachment domain) and may be involved in its turnover. Has angiogenic inhibitor activity.	[23,105–109]
ADAMTS5 (ADAM Metallopeptidase with Thrombospondin Type 1 Motif 5)	21q21.3	ECM-degrading enzyme that shows proteolytic activity towards the hyalectan group of chondroitin sulfate proteoglycans, including ACAN, VCAN, BCAN and NCAN. Plays an important role in connective tissue organization, development, inflammation and cell migration.	[23,109,110]
COL6A1 (Collagen Type VI Alpha 1 Chain)/COL6A2 (Collagen Type VI Alpha 2 Chain)	21q22.3/21q22.3	Collagen VI acts as a cell-binding protein. Collagen VI is a ubiquitous nonfibrillar collagen composed of three chains (α1(VI), α2(VI), and α3(VI)) organized into a network of microfibrils important in anchoring the basement membrane to the ECM. Each chain contains a comparatively short triple helical domain with repeating Gly-X-Y subunits flanked by large globular von Willebrand factor type A domains.	[23,28,29,33,109,111–114]
COL18A1 (Collagen Type XVIII Alpha 1 Chain)	21q22.3	Regulates ECM-dependent motility and morphogenesis of endothelial and non-endothelial cells. Inhibits angiogenesis by binding to the heparan sulfate proteoglycans involved in growth factor signaling. Inhibits VEGFA-induced endothelial cell proliferation and migration. Modulates endothelial cell migration in an integrin-dependent manner.	[23,109,115,116]
DSCAM (DS Cell Adhesion Molecule)	21q22.2	Plays a role in neuronal self-avoidance, promotes lamina-specific synaptic connections in the retina and mediates homophilic intercellular adhesion.	[14,27,33]
JAM2 (Junctional Adhesion Molecule 2)	21q21.3	Mediates heterotypic cell–cell interactions with its cognate receptor JAM3 to regulate different cellular processes.Plays a role in homing and mobilization of hematopoietic stem and progenitor cells within the bone marrow. Plays a central role in leukocytes extravasation by facilitating not only transmigration, but also tethering and rolling of leukocytes along the endothelium. During myogenesis, it is involved in myocyte fusion.	[23,117,118]
RUNX1 (Runt-Related Transcription Factor 1)	21q22.12	Required for the development of normal hematopoiesis. Forms the heterodimeric complex core-binding factor with CBFB. *RUNX* members modulate the transcription of their target genes. The heterodimers bind to the core site of several enhancers and promoters. Several studies have ascribed to RUNX1 an important role in regulating ECM genes, cell adhesion and migration.	[119–126]

Table 1. *Cont.*

Gene Name	Location	Gene Product	Potential Role in CHD
COL1A1 (Collagen Type I Alpha 1 Chain)/*COL1A2* (Collagen Type I Alpha 2 Chain)	17q21.33/7q21.3	Type I collagen is a member of group I collagen (fibrillar forming collagen). COL1A1 and COL1A2 genes provide instructions for making part of type I collagen. Type I collagen is the most abundant form of collagen in the human body.	[23,109,114,127]
COL3A1 (Collagen Type III Alpha 1 Chain)	2q32.2	Involved in regulation of cortical development, it is the major ligand of ADGRG1 in the developing brain. Binding to ADGRG1 inhibits neuronal migration and activates the RhoA pathway by coupling ADGRG1 to GNA13 and possibly GNA12.	[23,109,128,129]
COL5A1 (Collagen Type V Alpha 2 Chain)/*COL5A2* (Collagen Type V Alpha 2 Chain)	9q34.3/2q32.2	Type V collagen is a member of group I collagen (fibrillar-forming collagen). It is a minor connective tissue component of nearly ubiquitous distribution. Type V collagen binds to DNA, heparan sulfate, thrombospondin, heparin and insulin. Type V collagen is a key determinant in the assembly of tissue-specific matrices.	[23,109,114,130]
COL15A1 (Collagen Type XV Alpha 1 Chain)	9q22.33	Structural protein that stabilizes microvessels and muscle cells, both in heart and in skeletal muscle.	[23,109,131–133]
DDR1 (Discoidin Domain Receptor Tyrosine Kinase 1)	6p21.33	Functions as cell surface receptor for fibrillar collagen and regulates cell attachment to the ECM, remodeling of the ECM, cell migration, differentiation, survival and cell proliferation. Regulates remodeling of the ECM by upregulation of the metalloproteinases MMP2, MMP7 and MMP9, and thereby facilitates cell migration and wound healing.	[21]
DDR2 (Discoidin Domain Receptor Tyrosine Kinase 2)	1q23.3	Required for normal bone development. Functions as cell surface receptor for fibrillar collagen and regulates cell differentiation, remodeling of the ECM, cell migration and cell proliferation. Regulates remodeling of the ECM by upregulation of the collagenases MMP1, MMP2 and MMP13, and thereby facilitates cell migration and tumor cell invasion. Promotes fibroblast migration and proliferation, and thereby contributes to cutaneous wound healing.	[23,32]
FBLN1 (Fibulin 1)	22q13.31	Incorporated into fibronectin-containing matrix fibers. Plays a role in cell adhesion and migration along protein fibers within the ECM and contributes to the supramolecular organization of ECM architecture. Has been implicated in a role in cellular transformation and tumor invasion. Plays a role in hemostasis and thrombosis owing to its ability to bind fibrinogen and incorporate into clots.	[105,106,109,134,135]

Table 1. *Cont.*

Gene Name	Location	Gene Product	Potential Role in CHD
FN1 (Fibronectin 1)	2q35	Fibronectins bind cell surfaces and various compounds including collagen, fibrin, heparin, DNA and actin. Fibronectins are involved in cell adhesion, cell motility, opsonization, wound healing and maintenance of cell shape. Participates in the regulation of type I collagen deposition by osteoblasts.	[109,136–139]
VCAN (Versican)	5q14.2–q14.3	Plays a role in intercellular signaling and in connecting cells with the ECM. Takes part in the regulation of cell motility, growth and differentiation. Binds hyaluronic acid.	[106,107,109,140]

Aberrant cell adhesion, migration and proliferation have been demonstrated using a trans-chromosomic mouse model, which contains a supernumerary human chromosome 21 [141]. Possibly, increased adhesion and aberrant migration in DS cells are independent of each other; hence, they might be caused by different mechanisms and different Hsa21 genes, but both events might be required for the AVC defect to develop [141].

The increased adhesiveness of T21 cells has for many years been considered as a main cause for the failure of the embryonal endocardial cushion to septum fusion, which provokes the persistence of the AVC and/or the perimembranous VSD [142]. To better understand the molecular mechanisms, the integrin-mediated cell adhesive properties on FN, COLI and COLVI of skin fibroblasts isolated from DS and non-DS individuals have been compared [143]. All DS fibroblasts displayed an aberrantly increased adhesive capacity for COLVI if compared to non-DS fibroblasts with a mechanism dependent on the altered activation state of the β1 integrin [143].

Collagen VI is composed of three alpha chains, two of which are encoded by Hsa21 genes. It is a component of ECM responsible for anchoring cells within the three-dimensional tissue space by binding to cell surface integrins and other structural matrix components [144]. Its expression has been documented in the developing AV cushions and the adult AV valves of several species [29,112–114].

Collagen XVIII is highly expressed throughout the connective tissue core of the endocardial cushions and in forming the AV valve leaflets. It was closely associated with the EMT of endothelial cells into mesenchymal cushion tissue cells and was localized around these cells as they migrated into the cardiac jelly to form the initial connective tissue elements of the valve leaflets [116].

Other collagen proteins are expressed in the developing heart [109]. Collagen type I is normally expressed in AV valves and in the aortic wall and it is important for this vessel's elasticity and integrity [109]. Collagen type III is also expressed in the vasculature of mice. The *Col3a1* knockout mouse dies late in its adult life from the rupturing of blood vessels [128]. Recently, *COL3A1* was defined as the most common causative gene in a cohort of 121 CHD patients [129]. *Col5a1* knockout mice are embryonically lethal for cardiovascular insufficiency [130]. Finally, collagen type XV plays a role in matrix remodeling in the heart and participates in the organization of the collagen fibrils [133]. *Col15a1* knockout mice also revealed that it is required for proper circulation in specific microvascular beds. For these reasons, it should be considered as a candidate gene for involvement in human familial cardiomyopathies [133].

The Hsa21 gene *JAM2* encodes a cell membrane protein with immunoglobulin-like domains that is concentrated at cell-to-cell junctions in the heart endothelial cells of both large and small vessels, and it has been implicated in angiogenesis defects in Tc1 mice [117]. Jam2 plays a necessary role in the cross-talk between trisomy and Creld1 in Ts65Dn. Indeed, when present at 0, 1, 2, or 3 copies, it has no effect on heart development. However, when *Jam2* is trisomic and overexpressed, there is an increased penetrance of septal defects in mice with only one copy of *Creld1* [118].

VCAN may influence cell adhesion, proliferation, migration and survival by binding to several other ECM components. Its role is necessary for cardiac cushion formation, atrioventricular valve development, ventricular septation and OFT development [140]. VCAN is cleaved by MMPs and members of the ADAMTS family [106]. This process is necessary for AV cushion, OFT and trabecular development [106,107]. The dysregulation of ADAMTS proteins and MMPs is supposed to compromise this process.

FN1, a multi-domain ECM protein that interacts with multiple integrins, proteoglycans, collagens and fibrins [145], is expressed early in embryonic development [137,146]. As cardiac development progresses, it is expressed in the dorsal aortae, pharyngeal arch arteries, endocardium [137,139] and in the mesenchyme of the endocardial cushions, where it is required for EMT-mediated development [136,138].

FBLN1 can bind a variety of ECM molecules, including aggrecan and versican, which are known targets for cleavage by ECM metalloproteases [106,135,147]. At E9.0 to E9.5

in the mouse, fibulin-1 is expressed in the dorsal neural tube and is associated with the developing cardiac neural crest cells. By E10.5, fibulin-1 is found in pharyngeal arches 3 and 4 and the distal OFT cushions [134,135]. *FBLN1* is also expressed throughout the ECM of the AV cushions (ED9.5-ED14.5) [106]. It has been implicated in the regulation of cell migration of the AV mesenchyme [105], possibly due to its role as a cofactor to ADAMTS1, in the cleavage of ECM molecules known to promote migration [105,106].

4. The Hsa21 Transcription Factor RUNX1 Regulates the Expression of ECM Components

Searching for Hsa21 genes that may contribute to ECM gene regulation, RUNX1 (runt-related transcription factor 1) has been identified as a possible candidate according to the following evidence [148]: (i). the analysis of experiments in which *RUNX1* gene expression was modulated has shown that ECM is one of the most affected categories [149]; (ii). *RUNX1* and several ECM genes, located or not on Hsa21, are upregulated in human DS fetal hearts and fibroblasts [23], and most of them have consensus sequences for *RUNX1* in their promoters; (iii). the attenuation of RUNX1 by siRNAs decreased the expression of 11 out of 14 ECM genes that are overexpressed in DS fetal fibroblasts (*ADAMTS5*, *APP*, *COL6A1*, *COL6A2*, *COL18A1*, *COL5A1*, *ECM2*, *FN1*, *FBLN1*, *MMP2* and *VCAN*) and increased the migratory capacity of trisomic fibroblasts, which are characterized by a migration defect compared to euploid controls [148]; (iv). the *RUNX1* gene is included in the 3.7 Mb minimal critical region for DS-CHD, as described by Liu et al. [20].

The *RUNX* genes encode the α-subunits of a family of transcription factors that orchestrate proliferation, differentiation and cell survival in multiple lineages. In mammalian species, three α-subunits exist, known as RUNX1, RUNX2 and RUNX3, each with its own distinct spatial-temporal and tissue-specific pattern of expression [119,150]. Although each RUNX protein interacts with the same target consensus sequence, they display distinct and non-redundant biological functions [150]. In the developing embryo, RUNX1 is the most broadly expressed of all the RUNX proteins and is expressed in a range of tissues, including the mesenchymal tissue of the heart and in vascular tissue [119].

Several studies have ascribed to RUNX1 an important role in regulating ECM genes, cell adhesion and migration [151–154].

In the murine hemogenic endothelium, more than 100 genes were bound by RUNX1 and positively correlated with its expression. They were clearly associated with cell adhesion, such as α and β integrins, cellular movement and interaction with ECM, such as Adamts family genes and collagens. Overall, integrin signaling was the top enriched canonical pathway influenced by *Runx1* modulation [151]. Several genes encoding cell surface or extracellular ligands, involved in cell-matrix adherence, have been defined as candidates for direct Runx1 regulation [153]. Furthermore, *RUNX1* knockdown in breast cancer cells resulted in the downregulation of genes belonging to ECM-related categories. This correlation was confirmed by the presence of RUNX1 binding sites in dysregulated gene promoters [154].

Finally, transcriptome and proteome profiling of trisomic neural cells revealed the dysregulation of several genes belonging to collagen, cell-adhesion, ECM–receptor interactions and integrin complex clusters. The most consistent upregulation during differentiation was identified for *RUNX1* [155].

Genes belonging to the RUNX family are transcriptional factors for collagen proteins [156–158], including COLIV. Collagen IV protein levels were found to be increased in trisomic fibroblasts, and decreased upon RUNX1 attenuation [148]. The overexpression of RUNX1 in hepatocellular carcinoma cells elevated COL4A1 expression, while its knockdown in SMMC7721 and SK-Hep1 cells significantly decreased its expression level [159]. Collagen IV, the main component of the basement membrane in the heart, plays a major role in the cell–matrix interaction, thus regulating the cell differentiation, migration, proliferation, adhesion and signaling cascade [160].

The expression of *RUNX1* positively correlated with multiple molecules of the MMP family in colorectal cancer [161] and other human tumors [162–164].

Silencing of *RUNX1* expression demonstrated a significant decrease in the expression levels of *MMP1*, *MMP2*, *MMP9*, *MMP19* and *VEGFA* [164].

The importance of RUNX1 in the development of the vasculature is highlighted in the phenotype observed in *Runx1* KO mice (Table 1). In the heart, these mice have an underdeveloped coronary plexus and smaller ventricular free wall vessels [124]. This coincides with changes in the heart structure, including ventricular septal defects and the development of a thin myocardium [124].

The expression of *RUNX1* in the neonatal heart is higher compared with adult heart tissue [122]. Although the reasons for this are unexplored, it is interesting to note that genes with RUNX1-binding sites within their promoter region are over-represented in the collection of genes that become methylated during the first week of life [165]. In this setting, increased gene methylation may be important in the maturation process by switching off genes necessary for heart development to support transition to a more adult phenotype [125,166].

5. Conclusions

In this review we addressed the role of genes mapping to Hsa21 and other chromosomes in molecular mechanisms involved in CHD. The genes we discussed are linked by many different types of interactions (Figure 2), which may help to understand how they cooperate in the morphogenesis of the heart and the generation of its malformations.

The overexpression of Hsa21 genes is clearly responsible for the pathogenesis of CHD in DS, either directly or in an indirect manner, by dysregulating the expression of genes or miRNAs involved in embryonic cardiogenesis. A direct effect is exerted by the upregulation of Hsa21 genes, such as *DYRK1A* and *RCAN1*, which may affect septum morphogenesis by altering the NFAT signaling pathway; *DSCAM*, which increases cell adhesion; *COLVI*, which activates key ECM genes during cushion development; *NRIP1*, which controls mitochondrial function by regulating PGC-1α and the expression of NEMGs. The alteration of pathways resulting from the overexpression of these genes could be studied in an experimental model of T21 iPSCs [167], which can be differentiated into cardiomyocytes.

As an indirect effect, gene expression studies demonstrated in DS tissues and models the dysregulation of non-Hsa21 genes. The downregulation of genes belonging to the core network of cardiogenesis, such as *SRF*, *TBX20* and miR-1, and the global downregulation of genes encoding mitochondrial proteins—as well as the overexpression of genes encoding ECM proteins—have been documented in the heart of DS subjects [23].

The dysregulation and pathogenic mutations or polymorphisms of genes involved in cardiogenesis may concur to generate CHD. Rare variants of genes either belonging or associated to the VEGF-A pathway, as well as the CNV of Hsa21 tracts located within the previously identified DS-CHD region, have been found in DS subjects with AVSD, suggesting that they may increase the risk of CHD.

Quantitative and qualitative changes in the deposition of ECM molecules are crucial for tissue morphogenesis and homeostasis [168,169]. For this reason, the dysregulated expression and/or organization of ECM components in DS may be responsible for altered heart morphogenesis [29,170,171].

The overexpression of ECM-related genes in DS, postulated since 1998, when ultrastructural findings showed an extracellular precipitate containing glycosaminoglycans in the skin of DS human fetuses [172], was then demonstrated by gene expression profiling [23,102]. Specific changes in the expression and accumulation of ECM components have also been observed during human cardiomyocyte differentiation from trisomic embryonic stem cells in culture [140]. The ECM plays an important role in the development of the heart, in which the cardiac jelly, an acellular and ECM-rich space that separates the myocardial and endocardial cell layers in the primitive heart [173], is critical in heart septation and valvulogenesis [101]. It seems conceivable that even a small dysregulation of multiple ECM proteins involved in cardiogenesis may have profound effects on the proper formation of

the atrioventricular septum and outflow tract, resulting in cardiac defects such as those observed in DS.

It has been proposed and demonstrated that the upregulation of *RUNX1*, a gene mapping to Hsa21, contributes to the overexpression of ECM-related genes in trisomic cells and also accounts for the decreased migration of trisomic fibroblasts [148]. Although the expression of RUNX1 in the adult heart is reported to be low, several studies have demonstrated that RUNX1 expression is increased in the context of cardiac pathologies [120,121,123,126], suggesting a possible role of RUNX1 in cardiac remodeling after heart failure [126].

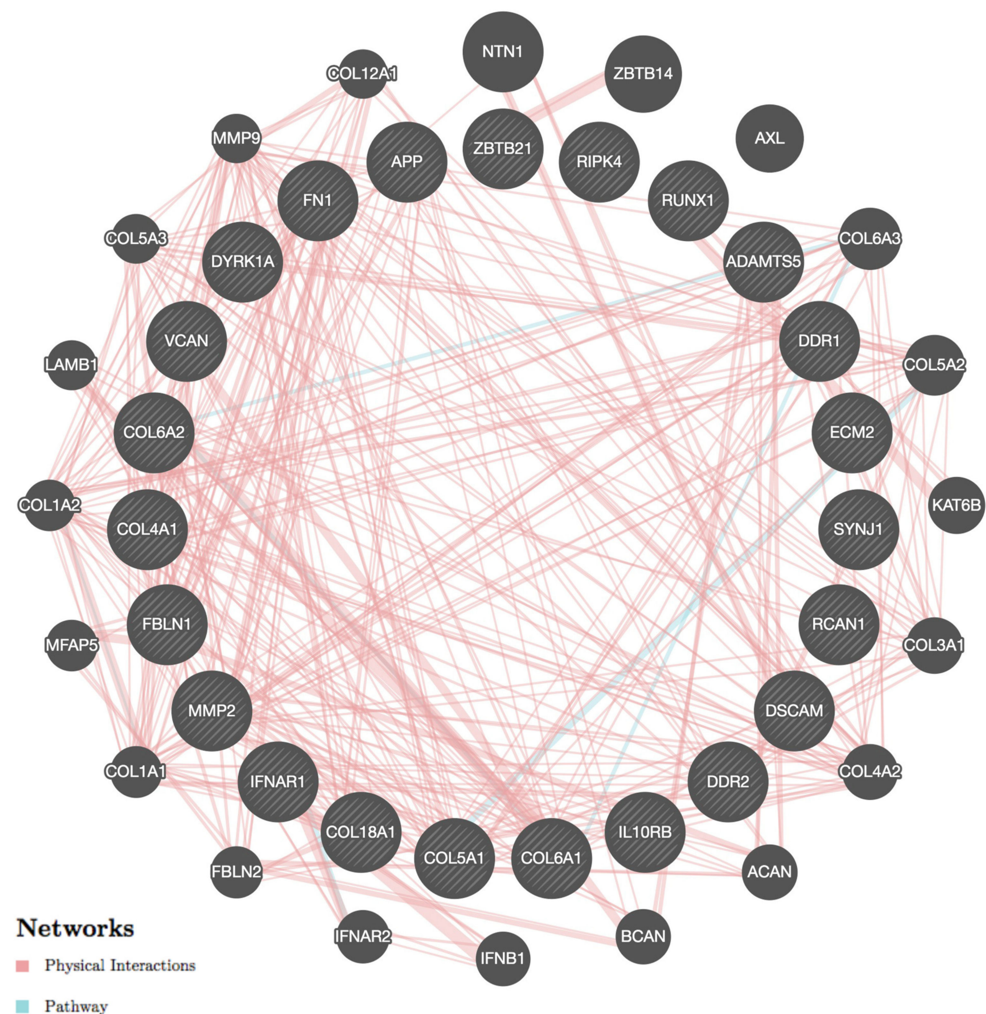

Figure 2. Interactions among genes involved in CHD. The figure illustrates the physical interactions and participation in the same pathway of genes involved in CHD generation, obtained using the GeneMANIA software (version 3.6.0) [174,175] (available at: https://genemania.org/ (accessed on 27 January 2023)). The genes discussed in this review are represented by the striped circles depicted in the inner ring. They are connected to each other by lines of different colors, each indicating a specific type of interaction: pink for physical interactions and light blue for pathways. The outer ring shows genes closely related to those in the inner ring (according to the GeneMANIA software). These genes are represented as circles of different sizes according to their ranking, which is achieved by scoring them using label propagation. By inputting the names of the genes in GeneMANIA, it is possible to generate an image featuring active links that provide additional information about all interactions and relevant literature references.

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