Intercalated Discs and Arrhythmogenic Cardiomyopathy

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Heart tissue is subjected to high mechanical stress. Different junctional complexes exist within the intercalated disc (ID) at the site of end-to-end contacts between cardiomyocytes. These junctions are essential for adhesive integrity, morphogenesis, differentiation, and maintenance of cardiac tissue. Recent findings of molecular interactions among intercellular adhesion molecules, gap junctions, and the voltage-gated sodium channel complex suggest that IDs should be considered an organelle in which macromolecular complexes interact specifically to maintain cardiac structure and cardiomyocyte synchrony. It is within this organelle that most of the mutated proteins involved in arrhythmogenic right ventricular cardiomyopathy (ARVC) reside. This inherited cardiomyopathy is characterized by both structural and electric abnormalities of the heart, particularly in young people and athletes. This review highlights recent advances in understanding the link between ID alterations and the molecular genetics and pathogenesis of ARVC.

Molecular Complexes at the IDs

Cardiomyocytes are extensively interconnected at their ends through their IDs, a complex region composed of different kinds of intercellular junctions essential for electric, mechanical, and signaling communication between adjacent cells and, hence, for maintaining correct heart function and growth. Although traditionally depicted as a composition of different separate units, recent data indicate that the ID of cardiomyocytes should be considered a single functional unit in which macromolecular complexes interact mechanically and electrically to maintain cardiomyocyte rigidity and synchrony.

Mechanical Junctional ID Components

The ID in vertebrates was originally described as consisting of 3 main junctional complexes: desmosomes, adherens junctions (AJ, also called fascia adherens in cardiac muscle), and gap junctions. It has been proposed that while gap junctions, being essential for chemical and electric coupling of neighboring cells, represent the electric component of ID, desmosomes together with AJ form the mechanical intercellular junctions in cardiomyocytes.[1] Desmosomes and AJ are highly specialized anchoring junctions. They are particularly important for maintenance of adhesion and integrity of tissues exposed to mechanical stress and show structures whose blueprints are comparable.[2] Both are composed of intercellular adhesion molecules connecting 2 adjacent cardiomyocytes by binding extracellularly like adhesion proteins extending from the adjacent cells; intracellularly, various adaptor proteins involved in signaling or linkage to the cytoskeleton.[3]

In the cardiac desmosome, desmoglein-2 and desmocollin-2, both transmembrane proteins of the cadherin family, mediate intercellular adhesion, and through their cytoplasmic domains, they serve as a scaffold for assembly of the desmosomal plaque (Figure 1). Indeed, these cytoplasmic domains provide a binding platform for the armadillo family members plakoglobin and plakophilin-2, which in turn associate with desmoplakin isoforms, which complete the link with desmin intermediate filaments (IF) through their C termini. This interaction of IF with desmosomes propagates the tensile strength imparted by the IF cytoskeleton across the entire tissue and is essential for myocardium integrity.[4,5] In the heart, AJ-like junctions consist of homodimers of N-cadherin, a classical cadherin that mediates intercellular adhesion through its extracellular domain, whereas its cytoplasmic tail is linked to so-called catenins of the armadillo protein family: p120ctn, β-catenin, and plakoglobin (Figure 1). The junctional role of β-catenin has been well studied particularly in epithelial cells: through interaction with αE-catenin, β-catenin forms a direct or indirect link with the F-actin cytoskeleton.

Accessory proteins, such as vinculin and EPLIN, are also involved in AJ. Together with αE-catenin, they confer mechanosensitive properties to these junctions.[6,7] It is unclear whether all those molecular interactions occur also in cardiomyocytes, but recent evidence suggests that also here AJ proteins are involved in mechanotransduction, the process of converting mechanical stimuli into biochemical signals and cytoskeletal remodeling. The cadherin–catenin complex seems to constitute an attachment site for myofibrils spanning adjacent cells and is thus essential for myofibril continuity across sarcolemma.[8,9] In particular, a direct role has been demonstrated for N-cadherin, not only in intercellular adhesion but also in bidirectional transmission of cytoskeletal tension between contractile cells.[10,11] In individual neonatal rat cardiomyocytes cultured on N-cadherin–coated Y-shaped micropatterns, αE-catenin localized to areas of high internal stress, but this was not the case when fibronectin-coated micropatterns were used.[12] This focal enrichment was disturbed when a myosin ATPase inhibitor was used. From these and similar

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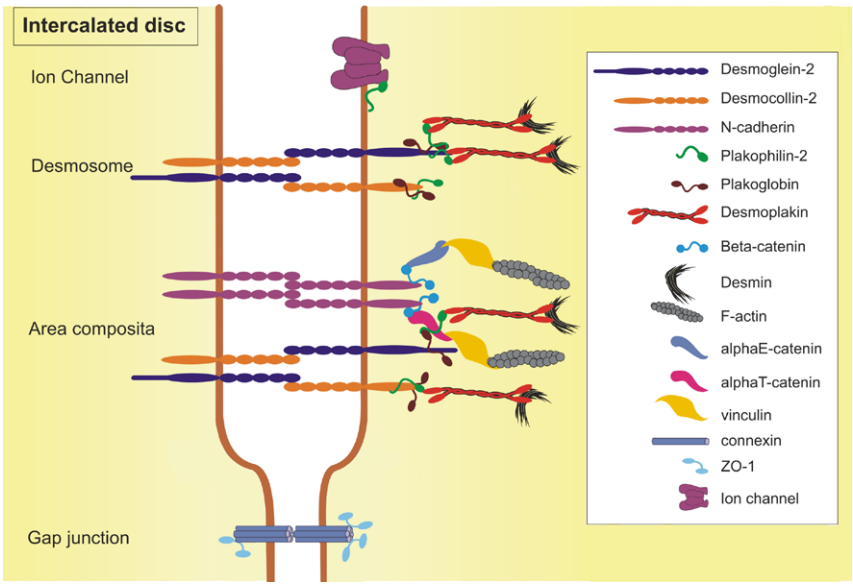

Figure 1. Molecular composition of the intercalated disc (ID) connecting cardiomyocytes. The various proteins are identified in the box on the **right**. Besides the ion channels, in particular, the cardiac sodium channel, 3 types of junctional complexes exist. Classical gap junctions are composed of connexins, mainly connexin-43 (Cx43) in the heart. Classical desmosomes are composed of desmosomal cadherins, which make intercellular connections and are connected via the armadillo proteins plakoglobin and plakophilin-2 to desmoplakin, which in turn recruits intermediate filaments of the desmin type. The third junction is the heart-specific area composita (AC), also called mixed-type adherens junction. A hallmark of the AC is the amalgamation of proteins typical of genuine desmosomes and of genuine adherens junctions (AJ), the latter being best studied in epithelial cells. Thus, in cardiac AC N-cadherin is extracellularly connected via β-catenin (or plakoglobin) to α-catenin, which in turn binds via vinculin to the F-actin cytoskeleton. However, in the heart, 2 forms of α-catenin are expressed: αE-catenin (which is widely expressed) and αT-catenin (which is enriched in the heart). αT-catenin has the unique capacity to bind also plakophilin-2, and this interaction is thought to be fundamental for the generation and stability of the AC. The relatively large size of the AC, and its combined linkage to both F-actin and intermediate filaments, is expected to provide the strong intercellular linkages necessary to support the mechanical stresses in heart tissue. As discussed in the text, arrhythmogenic right ventricular cardiomyopathy (ARVC) can be caused by mutations in many of the proteins depicted here, often in plakophilin-2 but also in αT-catenin. Moreover, the cross talk between the various molecular complexes shown here can be drastically distorted by the disease. Therefore, ARVC may be considered a disease of the ID, rather than a purely desmosomal disease.

experiments, it was concluded that the N-cadherin/αE-catenin complex regulates sarcomeric organization according to the mechanical stimulus and does so differently from integrin/vinculin complexes.

Area Composita: A Peculiar Junction at the ID

Interestingly, in vivo studies demonstrated that only in non-mammalian vertebrates and during fetal stages of mammalian development, AJ and desmosomes become uniformly distributed throughout the sarcolemma.[13,14] However, mammalian heart development continues postnatally with the polar clustering and amalgamation of AJ and desmosomal proteins into the ID.[14–16] By postnatal day 90, in mice, these junctional proteins are no longer restricted to distinct structures but exist almost completely in a hybrid and enforced structure, that is linked to both the actin cytoskeleton and the desmin IF.[16] Similar observations were made for cardiac IDs of other mammals, including man.[15–17] This ID-specific hybrid junction has been termed area composita (AC) (Figure 1).[14,15,17,18] Indeed, immunofluorescence and immunoelectron microscopy performed on myocardial samples of several mammalian species revealed in the AC the colocalization of different junctional proteins in more promiscuous assemblies than originally thought.[15,17] Apparently, in the same molecular complex, genuine desmosomal proteins, such as desmoplakin, the desmosomal cadherins, plakoglobin, and plakophilin-2, were observed in addition to N-cadherin, β-catenin, αE- and αT-catenins, p120ctn, myozap, and vinculin, all of which are components thought to be typical of cardiac AJ. The special mix of 2 major junctional ensembles and the resulting hybrid character of the AC are also underlined by the specific interaction in the myocardium of the desmosomal protein plakophilin-2 with αT-catenin, which shows high homology with αE-catenin.[19] The αE-catenin is a typical component of the AJ plaque, but it cannot bind plakophilin-2. Thus, the occurrence of a peculiar AC instead of an AJ at the ID could be a means of modulating and strengthening cell–cell adhesion between cardiac muscle cells.

In this novel view of the ID, most mechanical junctions at the ID appear as an extended, sometimes continuous system composed mainly of desmosomal proteins and AJ proteins intimately associated with each other. This AC occupies >90% of the ID area and is interrupted only by few gap junctions, genuine desmosomes, and rather small junction-free regions.[17] Junctions with desmosomal morphology occupy only a relatively minor proportion of the ID, often only ≤15%.[15,18]

Interestingly, the AC is not found in hearts of lower vertebrates, such as amphibian or fish species, in which desmosomes and AJ remain separate ensembles.[20] In avian hearts, only a small fraction of desmosomal proteins appear as AC. This suggests that the AC might have evolved to support the increased mechanical load on the mammalian heart because

it anchors at the ID both actin and IF cytoskeletons over an extended junctional area.[20,21] Taken together, these data indicate the importance of the AC in maintenance of the shape and the adhesion properties of cardiomyocytes in mammals, and thus in cardiac function in general.

Complex Links of the ID to the Cytoskeleton

In view of the essential role of the ID in intercellular adhesion and mechanical transduction, the binding of its various components to cytoskeletal elements and its functional implications should be scrutinized. As mentioned above, there is mounting evidence that α-catenins are more than linker molecules. In the AC of cardiomyocytes, 2 isoforms of α-catenins are expressed: αE- and αT-catenin.[19,22] At least for αE-catenin, it has been reported that it functions as a dynamic cytoskeleton modulator with tension-dependent junctional effects and nonjunctional effects.[6,7] Homodimeric forms of αE-catenin can inhibit Arp2/3-dependent actin polymerization, thereby preventing the formation of branched F-actin.[23,24] However, in epithelial cells, junctional αE-catenin can interact either with formin-1, leading to nucleation of unbranched actin filaments[25,26] or with EPLIN. This contributes to the assembly of mechanoresistant AJ.[27] One may wonder whether F-actin–modulating proteins with analogy to formin-1 or EPLIN are expressed in the myocardium, and if so, whether they show any specific interaction with one or both types of α-catenin at the ID. Moreover, the binding of αE-catenin, and possibly also αT-catenin, to the actin-binding proteins α-actinin and vinculin may also be important for local F-actin organization at the ID.[6,7] α-actinin is a component of cardiac Z-discs and might bind to α-catenins in the so-called cardiac transitional junction.[28] The latter region seems to link physically the highly ordered sarcomeric structures of the cardiomyocyte to the AC in the ID. Zygotic knockout of vinculin results in prenatal death as a result of severe defects in brain and heart.[29] It is also noteworthy that the heart expresses the formin family member Daam1, and abrogation of Daam1 by gene-trap technology was reported to cause multiple defects in the cytoskeletal architecture of the heart, including perturbation of the AC.[30]

Cross talk of Mechanical Junctions With Gap Junctions and Voltage-Gated Sodium Channels

The ID is composed of discrete molecular complexes (desmosomes, areae compositae, and gap junctions). Nonetheless, in recent years, several reports demonstrated that these structures belong to a communal network, the components of which interact synergistically at the cell–cell contacts. Molecules conventionally defined as belonging to 1 complex are also relevant to the function of the others.

It has been demonstrated that the mechanical junction protein plakophilin-2 and the gap-junction protein connexin-43 (Cx43) coexist in the same macromolecular complex because plakophilin-2 clusters have been found within the boundaries of the Cx43 plaque.[31,32] The relationship between these 2 molecules seems to extend to the functional level because shRNA-mediated knockdown of plakophilin-2 expression in rat ventricular cardiomyocytes led to a reduction in Cx43 amounts at intercellular contacts together with a decreased dye coupling between the cells.[31] This indicates that plakophilin-2 directly modulates Cx43. On the contrary, Cx43 has been demonstrated to be relevant to mechanical coupling, as shown by an elegant dispase assay performed in HEK293 cells by the group of Delmar.[33] However, whether this finding involves a physical interaction between Cx43 and mechanical junction proteins, or whether it is a consequence of intercellular adhesion mediated by gap junctions, remains to be determined.

Interactions at the ID have also been observed between mechanical junction proteins and several nonjunctional molecules. Sato et al[34,35] demonstrated that the voltage-gated sodium channel (NaV1.5) and ankyrin-G (AnkG), a scaffolding protein for the sodium channel, are involved in the binding and functional interaction with mechanical junction proteins. Indeed, NaV1.5 was found to coimmunoprecipitate with Cx43, N-cadherin, and plakophilin-2 and to coexist in apparently the same molecular complex at rat cardiac ID.[34] Furthermore, in neonatal rat cardiomyocytes with plakophilin-2 knockdown, the sodium current (I_{Na}) significantly decreased, and optical mapping experiments demonstrated increased re-entrant activity and significantly decreased conduction velocity when compared with control cardiomyocytes.[34] More recently, the authors demonstrated that knockdown of AnkG expression in neonatal rat cardiomyocytes led to significant changes in subcellular distribution and abundance of Cx43 and plakophilin-2, as well as a reduction in intercellular adhesion strength and electric coupling.[35] Reciprocal regulation of the abundance of AnkG and the localization of NaV1.5 by plakophilin-2 have also been demonstrated.[35] However, the precise contribution of AnkG to the overall adhesion strength of cardiomyocytes in situ is still unknown. Other groups, who used HL-1 cells or induced pluripotent stem cell (iPSC)–derived cardiomyocytes from a patient with plakophilin-2 deficiency, reported a correlation between plakophilin-2 deficiency and the reduction of I_{Na} amplitude, even in the absence of compromised cardiac structural integrity.[36,37] Similar results correlating mechanical junction protein abnormalities with reduction in I_{Na} amplitude have been observed also in vivo, both in mouse models with *Pkp2* haploinsufficiency or with cardiac overexpression of a mutated desmoglein-2, and in humans carrying desmoplakin mutations.[38–40]

Taken together, these data indicate that proteins of different intercellular structures are more closely linked than originally thought. This implies a more complex picture of the ID as a connexome, a molecular interaction network in which proteins of different junctional and nonjunctional complexes can regulate the functions of others, and together, control cardiac excitability, electric coupling, and intercellular adhesion.[41]

Changes in IDs Lead to ARVC

ARVC is a primary inherited myocardial disorder characterized by progressive cardiomyocyte death, followed by fatty or fibrofatty replacement.[42,43] ARVC is nowadays one of the leading causes of sudden cardiac death in young people and athletes and accounts for up to 10% of deaths from undiagnosed cardiac disease in people aged ≤65 years.[44] ARVC is usually diagnosed at the age of 20 to 40 years. Patients are

seldom ≤10 years although sporadic cases have been observed early in life, and even in the embryological phase, when the high dosage of some drugs may potentially contribute to its development.[45,46] ARVC prevalence is estimated at between 1:2000 and 1:5000,[47] and it affects men more frequently than women, with a 2.4:1 ratio.[48]

The most typical clinical presentation has 2 aspects: (1) electrocardiographic abnormalities, such as ventricular tachycardia with left bundle branch morphology, and T-wave inversion in the V1 to V3 leads and (2) functional and structural abnormalities mostly of the right ventricle, such as wall thinning, regional wall motion alterations, and global dilation. The disease is clinically heterogeneous, with interfamilial and intrafamilial variability, and its morbidity ranges from benign to malignant forms.[42,44] The broad phenotypic spectrum encompasses the right form as well as left-dominant and biventricular subtypes, and adoption of the more comprehensive term arrhythmogenic cardiomyopathy might be appropriate.[47]

Development of ARVC and its associated arrhythmic risk turned out to be influenced by exercise, which increases the risk of sudden cardiac death by 5-fold.[47] Recently, the first systematic human study on endurance athletes carrying ARVC causing mutations reported an association between exercise per year, clinical diagnosis, ventricular arrhythmias, and heart failure.[49] Furthermore, reducing exercise duration reduced arrhythmic risk and altered the clinical course of the disease.

Molecular Genetics of ARVC

Systematic evaluation of first- and second-degree relatives of affected probands suggests that up to 50% of ARVC cases are familial and follow an autosomal-dominant pattern of inheritance with incomplete penetrance and variable phenotypic expression.[43] The majority of disease-causing mutations detected in patients with ARVC occurs in genes encoding desmosomal and AC proteins (Table 1).[56,62] For the genes mentioned below, heterozygous mutations are commonly detected in patients with ARVC but homozygous mutations are rare.[74]

The first ARVC locus was mapped in 1994, at 14q23-q24, after evaluation of a large Venetian family.[75] It was only in 2000 that the first causal gene for an ARVC-associated recessive disorder, Naxos syndrome, was identified. This disease is characterized by palmoplantar keratoderma, woolly hair, and ARVC, and it is caused by a recessive mutation in the *JUP* gene, encoding plakoglobin.[50] A dominant mutation in *JUP* was detected in an ARVC family without cutaneous abnormalities and was found to affect plakoglobin stability at the junctions.[51] Genome-wide linkage analysis of a large Italian family showed for the first time that the desmoplakin gene (*DSP*) is the cause of the classic autosomal dominant ARVC form.[53] Many other homozygous and mostly heterozygous mutations in *DSP* have been detected in patients showing ARVC, and they were rarely combined with cutaneous abnormalities.[54,56]

Because plakoglobin and desmoplakin were known to be key proteins of desmosomes,[5] the focus of the gene hunt in ARVC was directed to genes encoding other desmosomal

Table 1. Human Genes Associated With ARVC

Gene	Chromosome Locus	Protein	Cellular Localization	ARVC Mutation Prevalence	Phenotype	References
Mechanical junctional ARVC genes						
JUP	17q21	Junction plakoglobin	Desmosome/area composita	Rare	ARVC, Naxos disease, DCM	50–52
DSP	6p24	Desmoplakin	Desmosome/area composita	6%–16%	ARVC, Carvajal disease, DCM	53–56
PKP2	12p11	Plakophilin-2	Desmosome/area composita	7%–70%	ARVC, DCM, Brugada syndrome	37,55–57
DSG2	18q12	Desmoglein-2	Desmosome/area composita	5%–25%	ARVC, DCM	56,58
DSC2	18q12	Desmocollin-2	Desmosome/area composita	Rare	ARVC, DCM	56,59–61
CTNNA3	10q21	αT-catenin	Area composita	Rare	ARVC	62
Other known ARVC genes						
RYR2	1q42-q43	Ryanodine receptor 2	Sarcoplasmic reticulum	Rare	ARVC, CPVT	63
TGFB3	14q23-q24	Transforming growth factor β3	Secreted	Rare	ARVC	64,65
TMEM43	3p25	Transmembrane protein 43 (TMEM43; LUMA)	Nuclear envelope	Rare	ARVC	66,67
DES	2q35	Desmin	Intermediate filaments	Rare	Overlap syndrome	68–70
TTN	2q31	Titin	Sarcomere	Rare	Overlap syndrome	71
LMNA	1q21.2-q21.3	Lamin A/C	Nuclear envelope	Rare	Overlap syndrome	72
PLN	6q22.1	Phospholamban	Sarcoplasmic reticulum	Rare	ARVC, DCM, overlap syndrome	73

ARVC indicates arrhythmogenic right ventricular cardiomyopathy; CPVT, catecholaminergic polymorphic ventricular tachycardia; and DCM, dilated cardiomyopathy.

proteins. *PKP2*, encoding plakophilin-2, is the most commonly mutated gene among patients with ARVC, with an estimated prevalence ranging from 7% to 51% and spikes of 70% (Table 1).[56,57] The varying mutation prevalence of *PKP2* gene in different cohorts might be because of the presence of founder mutations, the strictness with which diagnostic criteria are applied, the use of inconsistent definitions for pathogenicity, and geographical variations in genetic and nongenetic factors. Recently, different research groups detected in ARVC families heterozygous deletions of some *PKP2* exons and even of the entire *PKP2* gene, recurring with a frequency of 2%.[56,76–78]

On the basis of its mutation rate, the desmoglein-2 gene (*DSG2*), together with *DSP* and *PKP2*, belongs to the so-called 3 big ARVC genes. Heterozygous mutations in *DSG2* have been identified in patients with ARVC with a frequency range of 5% to 25% in different cohorts.[56,58] Pathogenic mutations were originally found in the desmocollin-2 gene (*DSC2*) by Syrris et al.[59] Later, only a few more mutations in this gene have been detected.[56,60,61]

In different cohorts, a significant proportion (4%–11%) of the patients were found to carry >1 mutation in the same or in different ARVC genes.[57,79–81] Compared with patients with a single ARVC mutation, carriers of multiple mutations exhibited more severe disease manifestations, such as higher prevalence of left ventricular involvement, major right ventricular dilatation, increased risk of lifetime major arrhythmic events, VT and syncope, or more frequent personal history of sudden cardiac death (aborted or not).[57,79,82,83]

Although the majority of ARVC mutations occurs in genes encoding ID proteins, few ARVC mutations have been detected in genes unrelated to intercellular junction complexes, such as *RYR2* (ryanodine receptor 2),[63] *TGFB3* (transforming growth factor β 3),[64,65] *TMEM43* (encoding the protein previously known as LUMA),[66] *DES* (desmin),[68–70] *TTN* (titin),[71] *LMNA* (lamin A/C),[72] and *PLN* (phospholamban).[73,84] Mutations in the *RYR2* gene have been shown to account for an atypical form of ARVC associated with polymorphic ventricular arrhythmias and for catecholaminergic polymorphic ventricular tachycardia, a peculiar malignant arrhythmic disease.[85] Most probably, the 2 diseases belong to the same nosographic entity.

Recently, using the candidate gene approach, our group identified *CTNNA3* as a novel ARVC gene.[62] This gene encodes αT-catenin, which binds plakophilin-2 and thereby contributes to the formation of the AC.[19,22] On the basis of the most recent description of the ID organization and the identification of this novel ARVC gene, we propose that ARVC may be considered a disease of the ID, rather than a purely desmosomal disease (Figure 2; Table 1).

Comprehensive mutation screening of known ARVC genes can detect causative mutations in ≈50% of probands,[44] suggesting that additional genes could be involved in the genetic determination of the disease. Several candidate genes encoding proteins related to cell–cell junctions were screened for mutations (Table 2), but negative results were obtained.[89–91] However, these studies were performed on a small number of patients with ARVC, suggesting the need to assess all these genes in large cohorts. Future research on ARVC genes should be focused on other components of cardiomyocyte adhesion, such as ARVCF and p120ctn,[18] as well as into components of pathways involved in ID junction assembly. Rho-family GTPases have a well-established and important role in E-cadherin–mediated cell–cell adhesion.[92] In skin keratinocytes, the Rho/Rho-kinase pathway has been shown to be necessary for normal desmosomal assembly.[93] Moreover, recent studies in keratinocytes demonstrated a cross talk between the Rho/Rho-kinase pathway and plakophilin-2 and plakoglobin.[94,95] These findings suggest that the Rho/Rho-kinase pathway might be equally important for assembly or stability of the cardiac ID although that has not been reported yet. Recently, a novel protein expressed at high levels in the heart, myozap, was identified as a component of the cytoplasmic plaques of the AC in the myocardial ID.[96] Interestingly, myozap can interact with desmoplakin, ZO-1, and dysbindin. The latter is also strongly expressed in the heart and is an interaction partner of RhoA,[97] whereas myozap interacts with myosin phosphatase-RhoA interacting protein, a negative regulator of Rho activity.[96] Both myozap and dysbindin contribute in a RhoA-dependent way to activation of the transcription factor SRF (serum response factor). Myozap inhibition in zebrafish, as well as overexpression of dysbindin in cultured rat cardiomyocytes and cardiac-restricted overexpression of myozap in transgenic mice, induces various forms of cardiomyopathy but no ARVC-like phenotype.[96–98]

Genetic screening has been assuming an important role in clinical evaluation. It allows interpretation of borderline clinical phenotypes and early identification of asymptomatic carriers. Genetic testing is especially useful in families with ≥1 affected member who carries a pathogenic mutation because it allows presymptomatic diagnosis among relatives. Symptom-free carriers need lifelong clinical assessment, because the disease is progressive and can appear late in life. Restriction of physical exercise,

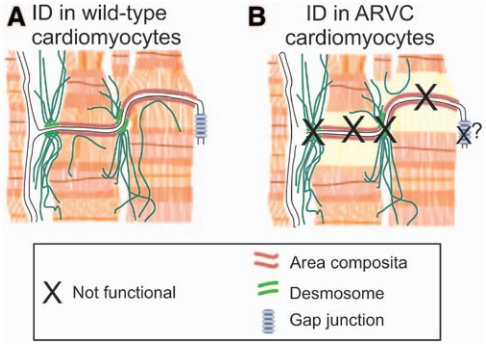

Figure 2. Scheme of junctional structures in (**A**) normal intercalated discs (IDs) of adult mammalian heart and in (**B**) arrhythmogenic right ventricular cardiomyopathy (ARVC) pathological heart caused by mutations in ID components. **A**, Typical composition of ID-associated junctions in healthy heart. Depicted are desmosomes (green), areae compositae (red), and gap junctions (blue). Black lines: cardiac sarcolemma; dark green filaments: desmin-containing intermediate filaments; light orange filaments: actin-rich myofilaments; dark orange filaments: myosin-rich myofilaments. **B**, IDs of ARVC heart in patients with mutations in genes encoding area composita or desmosomal proteins, which are expected to affect all 3 intercellular junction types. Remodeling of cardiac gap junctions and pale areas lacking filaments adjacent to the IDs are frequently observed in ARVC-afflicted hearts.[86–88] The negative effect on each of the 3 intercellular junction types at the IDs results in ARVC pathology. Modified after Pieperhoff et al.[18]

Table 2. Candidate Genes Screened for Mutations in ARVC Probands

Gene	Chromosome Locus	Protein	Cellular Localization	No. of ARVC Probands Screened	References
PKP4	2q24.1	Plakophilin-4	Desmosome	64	89
CTNNB1	3p21	Beta-catenin	Area composita	65	90
MYL3	3p21.31	Myosin light chain 3	Sarcomere	14	91
CTNNA1	5q31.2	AlphaE-catenin	Area composita	14	91
GJA1	6q22.31	Connexin 43	Gap junction	14	91
PERP	6q24	Perp	Desmosome	64, 65	89, 90
CAV1	7q31	Caveolin-1	Caveolae	64	89
MVCL	10q22.2	Metavinculin	Contractile apparatus	14	91
MYL2	12q24.11	Myosin light chain 2	Sarcomere	14	91
PNN	14q21.1	Pinin	Desmosome associated	64	89
ACTC1	15q14	Actin alpha cardiac muscle 1	Sarcomere	14	91
CDH2	18q12.1	N-cadherin	Area composita	14	91

antiarrhythmic drug and β-blocker therapy, and implantable cardioverter-defibrillator are effective life-saving treatments that can change the natural history of this disease and improve the expected quality of life of mutation carriers. However, genotype–phenotype correlation studies failed to identify specific genes or mutations distinctively associated with an unfavorable arrhythmic outcome in ARVC. To date, there is little evidence to support the use of genetic screening for risk stratification and optimization of therapeutic strategies.[47]

Although genetic testing has played a key role in studying disease expression, it cannot explain the phenotypic variability even among family members sharing the same mutation, thus suggesting cross talk interactions between genetic backgrounds and environmental factors. Modifier genes and common sequence variants, as well as environmental and endogenous factors (such as age, sex, strenuous exercise, drugs, hormones, infection/inflammation, and emotional stress), could account for much of the variation between individuals.[99]

Morphological Analysis of the IDs in ARVC

ARVC cardiomyocytes have been rarely investigated at the ultrastructural level. In the first studies on endomyocardial biopsy specimens, ARVC cardiomyocytes showed breaks in the sarcolemma, thickening of basal lamina, unusual presence of fibrillar material inside the T-tubules, and altered mitochondria.[100,101] More specifically, in 1989, Guiraudon[102] first showed that IDs may present pale structure and flattened convolutions with rare or small desmosomes and decreased filaments in the area of fascia adherens, but few cases of ARVC were investigated and quantitative data were not provided.

Interest in the microscopic analysis of IDs in ARVC increased on identification in patients with ARVC of mutated genes encoding ID proteins. The major ultrastructural changes reported for IDs in ARVC endomyocardial biopsies were the presence of abnormally small junctions composed of series of short desmosomes, as well as the presence of elongated desmosomes often located abnormally, and a decreased number of desmosomes per unit tissue area.[86] Moreover, although the IDs showed a normal membrane convolution in these ARVC samples, pale cytoplasmic plaques were often seen, but it is unknown whether these pale regions belong to defective desmosomes, abnormally organized AC, or both. As these ID abnormalities were observed in patients with ARVC with or without mutations in *DSG2*, *DSP*, or *PKP2*, this indicates that disease might also be caused by unknown ID component mutations.[86] Interestingly, ultrastructural changes in cardiac myocytes of Boxer dogs with spontaneous ARVC included reduced numbers of AJ (likely to be renamed AC) and gap junctions, whereas desmosomes were either fewer (right ventricle) or shortened (left ventricle).[88] Remarkably, pale areas lacking filaments were seen adjacent to the IDs of the ARVC-afflicted samples; more specifically, a wider gap separated the end of sarcomeric actin filaments from the ID membrane. This is indicative of a disrupted interaction of cytoskeletal structures with the AC. Unfortunately, despite reduced expression of plakoglobin, plakophilin-2, desmoplakin, and connexin-43 at the IDs of such ARVC-afflicted dogs, no mutations in the corresponding genes were detected.[103] The status of α-catenins in ARVC dog hearts was not reported.

Electron microscopy analyses of samples from an early stage patient with Naxos disease, caused by a recessive mutation in the *JUP* gene, revealed 2 to 5× smaller and 1.5 to 4× fewer gap junctions interconnecting myocytes in both left and right ventricles when compared with left ventricular control samples.[104] Knockdown of plakophilin-2 expression in neonatal rat ventricular myocytes affected total connexin-43 levels, induced redistribution of connexin-43 to the cytoplasm, and interrupted gap junction-mediated coupling between pairs of myocytes.[31] A study on human ARVC myocardial samples revealed that dominant mutations in various desmosomal proteins were consistently associated with marked reduction of plakoglobin and connexin-43 in the IDs.[87] This evidence of remodeling of cardiac gap junctions in patients with ARVC carrying desmosomal gene mutations suggests that arrhythmogenic substrate may arise from junctional cross talk between desmosome or AC proteins, on the one hand, and gap junction proteins at the ID, on the other hand. Most recently, immunocytochemical assays of affected heart

samples showed decreased levels of the cardiac sodium channel NaV1.5, in addition to a reduction and mislocalization of Cx43 and plakoglobin at the IDs.[105] These data suggest that abnormalities in NaV1.5 might also contribute to arrhythmia vulnerability in patients with ARVC.

Further morphological evidence for this wider cross talk comes from studies performed on ARVC animal models. Rizzo et al[39] dissected the early stages of ARVC development in transgenic mice with cardiac overexpression of desmoglein-2 carrying the N271S missense mutation. They observed a widening of the intercellular space in the ID and a reduction in action potential upstroke velocity caused by lower Na^+ current density. This supports a model of slowed conduction and increased arrhythmia susceptibility as soon as ID remodeling occurs, and before onset of necrosis and replacement fibrosis.

Several components have been implicated in the molecular basis of ARVC, and it has become clear that the disease is associated with a reduction in intercellular coupling, and probably also in membrane excitability. However, still unresolved are the extent to which these components contribute to the highly arrhythmogenic phenotype in ARVC and the exact sequence of events that lead to structural and functional disruption of the macromolecular complex at the ID.

Molecular Pathogenesis of ARVC

Despite growing knowledge on the genetic basis of ARVC, early molecular events leading to cardiomyocyte degeneration, fibrosis, and adipose substitution remain unknown. In addition to the inactivation of junctional mechanical functions, which can lead to myocyte death under physical stress, the suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin translocation has been suggested to promote adipogenesis in mesodermal precursors (Figure 3).[106] The first indication that a defect in canonical Wnt signaling is involved in ARVC came from in vitro studies in the HL-1 mouse cardiac muscle cell line. Stable transfection of HL-1 cells with siRNA to suppress desmoplakin expression specifically was associated with translocation of plakoglobin to the nucleus and a 2-fold reduction in signaling through the canonical Wnt/β-catenin/Tcf/Lef pathway.[107] This pathway is known to regulate adipogenesis, fibrogenesis, and apoptosis, and expression of genes related to these processes seemed to be increased in cells and mice deficient in desmoplakin.[107] To assess the cellular origin of excess adipocytes in ARVC, lineage tracer mice were generated in which *Dsp* was heterozygously ablated and, concomitantly, enhanced yellow fluorescent protein was expressed under control of different cardiac lineage promoters, including the second heart field-specific marker *Mef2C* promoter.[106] Results from these and related experiments suggest that most adipocytes observed in the myocardium of $Dsp^{+/-}$ mice originate from the second heart field cardiac progenitors, which switch to adipogenesis because the increased nuclear plakoglobin suppresses canonical Wnt signaling.[106]

In a conditional mouse model for ARVC based on cardiac tissue–restricted deletion of *JUP*, disruption of junctional

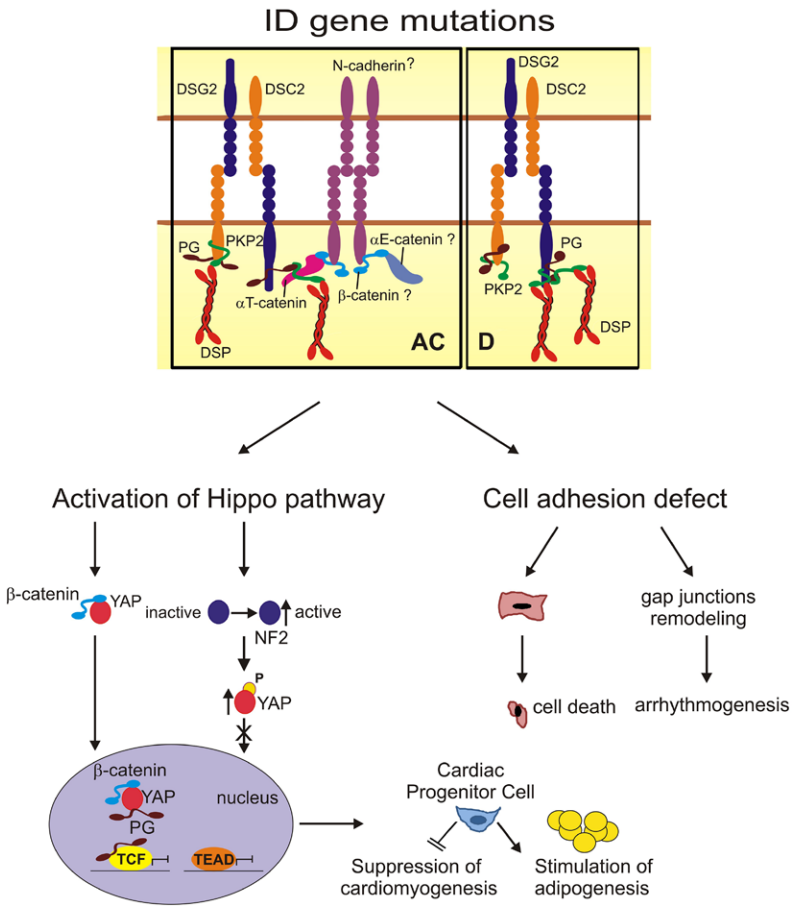

Figure 3. Model of the molecular pathogenesis of arrhythmogenic right ventricular cardiomyopathy (ARVC). **Top**, the ID with its 2 major junctions: area composita (AC) and desmosomes (D; see also Figure 1). Annotated are ID proteins that have been found to be mutated in ARVC (see also Table 1) or have been proposed as candidate ARVC proteins (indicated by question mark; see also Table 2). Mutant ID proteins can cause cell adhesion defects, which have various consequences on heart physiology (**right bottom**). Such mutations could provoke loss of membrane integrity and induce cell death; moreover, altered mechanical junctions can lead to gap junction remodeling, causing ventricular arrhythmias. On the contrary (**left bottom**), junctional remodeling reduces protein kinase Cα levels and activates neurofibromin 2 (NF2 or merlin), which acts upstream of the Hippo signaling pathway. This eventually leads to phosphorylation and cytoplasmic retention of the transcriptional coactivator Yes-associated protein (YAP). Cytoplasmic YAP forms a complex with β-catenin and thus sequesters this key protein outside the nucleus. However, junctional distortion can free plakoglobin, which enters the nucleus and competes there with any remaining β-catenin. Consequently, gene expression mediated by members of the SV40 transcriptional enhancer factor domain (TEAD) and families (effectors of the Hippo and canonical Wnt pathways, respectively) is reduced, resulting in increased adipogenesis in the ARVC heart. It is thought that most adipocytes originate from the second heart field cardiac progenitors, which switch to adipogenesis upon suppression of canonical Wnt signaling (see text for further details and references). DSC2 indicates desmocollin-2; DSG2, desmoglein-2; DSP, desmoplakin; PG, plakoglobin; and PKP2, plakophilin-2.

integrity caused increased β-catenin stabilization, apparently as a result of activation of AKT, what in turn inhibits glycogen synthase kinase-3β.[108] However, this hypothesis of activated β-catenin signaling in ARVC was not confirmed in another *Jup* conditional knockout mouse model.[109] Remarkably, in the latter model, β-catenin levels were increased at IDs of the *Jup* mutant cardiomyocytes, but Wnt/β-catenin–mediated signaling was not activated, whereas TGFβ signaling was upregulated during the early stages of cardiomyopathy.[109] TGFβ signaling induces myocyte cell death, including both apoptosis and necrosis, and influences robustly cardiac fibrosis and hypertrophy. In a third study, c-Kit$^+$ cardiac progenitor cells from plakoglobin-null mouse embryos were resistant to adipogenesis and expressed canonical Wnt signaling target genes.[110] Moreover, transgenic mice overexpressing either wild-type plakoglobin or an ARVC-causing truncated plakoglobin in the cardiac lineage exhibited an ARVC-like phenotype. The transgene-encoded plakoglobin was enriched in the nucleus and the transgenic cardiac progenitor cells showed enhanced adipogenesis, which could be prevented by drug activation of canonical Wnt signaling.[110]

Most recently, desmosome disruption in ARVC was linked to activation of the Hippo/Yes-associated protein (YAP) pathway.[111] In normal cardiomyocytes, active Hippo signaling leads to phosphorylation and cytoplasmic retention of the transcriptional coactivator YAP, and this restricts cardiomyocyte proliferation and thus heart size. If Hippo signaling is inhibited, YAP enters the nucleus and associates with SV40 transcriptional enhancer factor domain (TEAD) and β-catenin/T-cell factor (TCF) complexes, leading to enforced transcriptional activity of both TEAD and TCF, which are positive regulators of cardiac growth.[112,113] To examine what happens in ARVC hearts, Chen et al[111] investigated both Hippo/YAP and Wnt/β-catenin signaling pathways in human ARVC samples, 2 ARVC mouse models, and plakophilin-2 knockdown HL-1 myocytes. Their data indicate that the junctional remodeling reduces protein kinase C-α activity, which in turn activates NF2 (Merlin), which in the end leads to phosphorylation and cytoplasmic retention of YAP.[111] As this YAP form sequesters β-catenin and plakoglobin in the cytoplasm, both β-catenin/TCF and YAP/TEAD transcriptional activities are reduced, resulting in increased adipogenesis in the ARVC heart. These findings thus suggest a novel explanation: molecular changes at the IDs in patients with ARVC carrying mutations in genes encoding ID proteins modulate the cross talk between Wnt/β-catenin and Hippo/YAP signaling pathways (Figure 3). Levels of several ID proteins, including plakoglobin, are markedly reduced in ARVC human hearts, suggesting that the assembly of the IDs requires coordinated interactions between its protein constituents.[87] Impaired ID assembly is expected not only to affect the mechanical integrity of myocyte–myocyte attachment but also to promote a series of signaling events that are regulated at the IDs (Figure 3).

Studies on iPSC-Derived Cardiomyocytes

Transgenic animal models have been instrumental in enhancing ARVC pathogenesis understanding, but species differences in cardiac electrophysiological properties may limit our comprehension of human disease pathogenesis. A new appealing tool to improve this understanding is the differentiation of patient-specific iPSCs into cardiomyocytes, which could be used to recapitulate ARVC features in the context of the patient's genetic background.[114]

Cardiomyocytes derived from ARVC–iPSCs showed, in comparison with control cardiomyocytes, markedly reduced immunofluorescent signals of plakophilin-2 and plakoglobin, but similar levels of staining for N-cadherin, desmoplakin, and connexin-43.[115] Moreover, transmission electron microscopy revealed that ARVC–iPSC cardiomyocytes are larger and contain darker lipid droplets compared with controls, thus suggesting that these cells have increased adipogenic potential. Other experiments with iPSC-cardiomyocytes carrying a homozygous or heterozygous *PKP2* frameshift mutation showed, under baseline cardiogenic conditions, nuclear translocation of plakoglobin and low β-catenin expression and activity,[36] in line with the above-mentioned ARVC animal models.[107] Moreover, by culturing beating mutant embryoid bodies in a lipogenic milieu, Kim et al[36] observed a significant increase in the expression of the master proadipogenic transcription factor peroxisome proliferator-activated receptor alpha, and an abnormally hyperactivated peroxisome proliferator-activated receptor alpha pathway. This coactivation led to a pathology typical of ARVC: lipogenesis, apoptosis, and calcium-handling deficits. These important findings have been confirmed in a recent study on iPSC-derived cardiomyocytes from 2 ARVC patients carrying heterozygous *PKP2* frameshift mutations.[116] In cardiomyocytes displaying more severely distorted desmosomes, clusters of lipid droplets were associated with abnormal upregulation of proadipogenic peroxisome proliferator-activated receptor alpha, and this could be prevented by a glycogen synthase kinase-3β inhibitor known to promote Wnt/β-catenin signaling. Contrary to other studies on iPSC-derived cardiomyocytes, this study included ultrastructural characterization: desmosomes were severely distorted in the mutant cardiomyocytes and this distortion correlated with lipid accumulation.[116] It was not mentioned by the authors, but the ACs might also have been strongly affected.

Additional studies using iPSC-derived cardiomyocytes, including those generated from other types of patients with ARVC harboring alternative ID protein mutations are required to determine the true potential of this approach in providing additional insights into this disease.

Conclusions

The identification of ARVC-causing mutations in *JUP*, *DSP*, *PKP2*, *DSG2*, and *DSC2* led to the idea that desmosomal dysfunctions could be the common pathway leading directly to the pathogenesis of ARVC. But 2 developments could change this view. One is the recent description of the AC in mammalian cardiomyocytes as a large hybrid structure composed of both desmosomal and adherens junctional proteins. The other is the observed extensive cross talk between molecular complexes within the ID, which were previously thought to be independent. Thus, to understand the molecular pathophysiology of ARVC, one should consider the overall functional unit of the ID.

Evidence is emerging that it is essential to choose appropriate cell systems in which the interactions between mechanical and electric components of the ID can be studied. Only in

this way it will be possible to dissect the sequence of events, leading to disruption of the ID and to the electric and structural changes typical of ARVC. iPSC-derived cardiomyocytes are not an in vivo model and their predictive value for individualized management of patients is still far from proven. Nevertheless, they could be a most useful human-based platform to study the biology of ARVC, to develop drug toxicity screening assays, and to generate new therapeutic possibilities. The challenge for the future advancement of the ARVC field would be to realize such extensive studies.

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Disclosures

None.

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KEY WORDS: arrhythmogenic right ventricular dysplasia-cardiomyopathy ■ arrhythmias ■ cell adhesion molecules ■ mutation ■ molecular biology

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