Cardiac mesenchymal stromal cells are a source of adipocytes in arrhythmogenic cardiomyopathy

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Aim

Arrhythmogenic cardiomyopathy (ACM) is a genetic disorder mainly due to mutations in desmosomal genes, characterized by progressive fibro-adipose replacement of the myocardium, arrhythmias, and sudden death. It is still unclear which cell type is responsible for fibro-adipose substitution and which molecular mechanisms lead to this structural change. Cardiac mesenchymal stromal cells (C-MSC) are the most abundant cells in the heart, with propensity to differentiate into several cell types, including adipocytes, and their role in ACM is unknown. The aim of the present study was to investigate whether C-MSC contributed to excess adipocytes in patients with ACM.

Methods and results

We found that, in ACM patients’ explanted heart sections, cells actively differentiating into adipocytes are of mesenchymal origin. Therefore, we isolated C-MSC from endomyocardial biopsies of ACM and from not affected by arrhythmogenic cardiomyopathy (NON-ACM) (control) patients. We found that both ACM and control C-MSC express desmosomal genes, with ACM C-MSC showing lower expression of plakophilin (PKP2) protein vs. controls. Arrhythmogenic cardiomyopathy C-MSC cultured in adipogenic medium accumulated more lipid droplets than controls. Accordingly, the expression of adipogenic genes was higher in ACM vs. NON-ACM C-MSC, while expression of cell cycle and anti-adipogenic genes was lower. Both lipid accumulation and transcription reprogramming were dependent on PKP2 deficiency.

Conclusions

Cardiac mesenchymal stromal cells contribute to the adipogenic substitution observed in ACM patients’ hearts. Moreover, C-MSC from ACM patients recapitulate the features of ACM adipogenesis, representing a novel, scalable, patient-specific in vitro tool for future mechanistic studies.

Keywords

Arrhythmogenic cardiomyopathy • Mesenchymal stromal cells • Adipogenesis • Fibrofatty substitution • Plakophilin2 • Plakoglobin
**Translational perspective**

Fibro-adipose substitution has a double detrimental effect on the myocardium in arrhythmogenic cardiomyopathy (ACM), worsening arrhythmogenesis by creating a non-conductive substrate, and causing ventricular dysfunction leading to heart failure. Notably, to-date no etiological therapy is available. This work introduces, for the first time, the stromal cardiac compartment as a key player in ACM ventricular adipose substitution: we demonstrated that cardiac human mesenchymal stromal cells undergo adipogenic differentiation both in ACM explanted hearts and in culture through a PKP2-dependent mechanism. Cardiac mesenchymal stromal cells constitute a suitable cellular platform for future mechanistic studies and a potential target for future therapies.

**Introduction**

Arrhythmogenic cardiomyopathy (ACM) is a genetic disease in which the ventricular myocardium, predominantly the right ventricle, is progressively substituted by fibro-adipose tissue. Arrhythmogenic cardiomyopathy is associated with severe ventricular arrhythmias, sudden death, and progressive heart failure.

Fibro-adiposis is the hallmark of the disease; it typically starts from the epicardium at the ‘triangle of dysplasia’ and progresses toward the endocardium. Arrhythmogenic cardiomyopathy has a double pathologic phenotype: arrhythmias and ventricular dysfunction. The pathogenic mechanisms for both phenotypes are unclear. Although a primary electrical cause of arrhythmias involving sodium channels and connexins has been proposed, fibro-fatty substitution may worsen the condition of patients’ by creating non-conducting tissue. Moreover, progressive adipocyte deposition leads to loss of cardiac contractility and ultimately heart failure.

The disease usually has an autosomal dominant transmission with variable penetrance and expressivity. Rare recessive forms of ACM occur in conjunction with skin manifestations. Approximately 50% of symptomatic individuals harbour a mutation in one of the genes of the five major members of the cardiac desmosome: DSP (desmoplakin), PG (plakoglobin), PKP2 (plakophilin2), DSG2 (desmoglein2), and DSC2 (desmocollin2). Desmosomes are complex cellular junctions found mainly in epidermal cells and in cardiac myocytes. Non-desmosomal genes TGFβ3, TMEM43, LMNA, DES, TTN, PLN, CTNNA3, RYR2, and a number of other loci have also been associated with ACM.

It has been suggested that defective desmosomes cause stretch-induced cardiomyocyte death and impair intracellular signaling that determines cell fate. As a consequence, a cell differentiation hypothesis has been formulated to explain the fibro-adipose replacement, involving the inhibition of canonical WNT/β-catenin signaling, an important regulator of myogenesis vs. adipogenesis.

A key question in ACM pathogenesis is the cellular origin of excess adipocytes. The signal for enhanced fibro-adipogenesis is likely to originate from cells that express desmosomes. Mature cardiomyocytes, second heart field cardiovascular progenitor cells (c-KIT/SCA1), or sub-epicardial progenitor cells are the most extensively studied candidates.

Mesenchymal stromal cells (MSC) are supportive cells found in many tissues. They are multipotent and, under appropriate stimuli, can differentiate into osteoblasts, chondroblasts, and adipocytes. Mesenchymal stromal cells are characterized by the expression of mesenchymal surface antigens such as CD44, CD105, CD29, and CD90. In our laboratory, a population of cardiac mesenchymal stromal cells (C-MSC) isolated from adult human auricles have been characterized. In a normal adult heart, cardiomyocytes only represent 30% of the total cell number. The remaining 70% consists of cells other than cardiomyocytes, among which C-MSC are the vast majority. Cardiac mesenchymal stromal cells play a critical role in maintaining normal cardiac function, as well as in cardiac remodelling during pathological conditions. Mesenchymal stromal cells switch between osteoblast and adipocyte differentiation has been reported as a causative mechanism of osteoporosis.

The present study addresses, for the first time, the role of C-MSC in ACM adipose substitution in human hearts. We provide evidence that in ACM hearts cells differentiating into adipocytes express mesenchymal markers and C-MSC isolated from human ACM hearts express desmosomal genes and are more prone to accumulate fat (lipogenesis) and specifically differentiate into adipocytes compared with C-MSC from control hearts.

**Methods**

Detailed/additional methods are presented in Supplementary material online.

**Ethical statement**

This study complies with the declaration of Helsinki. Arrhythmogenic cardiomyopathy (ACM) patients’ samples use for research was approved by Centro Cardiologico Monzino and La Sapienza University Ethic Committees. Samples from patients not affected by arrhythmogenic cardiomyopathy (NON-ACM) were obtained from cadaveric donors from Fondazione Banca dei Tessuti di Treviso.

**Cardiac mesenchymal stromal cells culture and adipogenic differentiation**

Cardiac mesenchymal stromal cells were isolated, cultured, and characterized as previously reported. Adipogenic differentiation was obtained using adipogenic medium for 72 h or 1 week. Fat accumulation was tested by Oil Red O (ORO) or Nile Red staining; adipogenesis was tested by anti-Perilipin1 (PLIN1) antibody staining.

**Gene expression analysis**

qRT-PCR was performed in duplicate using 10 ng cDNA obtained after total RNA extraction with TRIzol, treatment with Dnase I, and reverse transcription with Superscript III according to manufacturer’s instructions. Primer sequences are reported in Supplementary material online, Table S4.

**Protein expression analysis**

Western blots were performed by standard procedures, after total protein extraction by Laemmli buffer lysis, target proteins were detected with
<table>
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<th>Sample type</th>
<th>ACM patient ID</th>
<th>Sex</th>
<th>Age</th>
<th>Type/age of first manifestation</th>
<th>Dysfunction and structural alterations</th>
<th>Tissue characterization of wall</th>
<th>Repolarization ab.</th>
<th>Depolarization/ conduction ab.</th>
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<td>VT/34</td>
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<td>H2  F 37</td>
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FFPE, formalin-fixed, paraffin-embedded tissue section; Echo, 2D-echocardiography; CMR, cardiac magnetic resonance; AG, angiography; n.a., not available; PVC, premature ventricular contractions; VT, ventricular tachycardia; EH, explanted heart; neg., negative; ab., abnormalities.

*C Only PKP2 was examined.*
primary antibodies PKP2, PG, DSP1/2, DSG2, DSC2, PPARy, PLIN1, FABP4, and GAPDH (see Supplementary material online, Table S3).

Plakophilin2 overexpression
Cardiac mesenchymal stromal cells were transfected with control GFP or wild-type PKP2-GFP vector\textsuperscript{30} using the Neon Transfection System.

Cardiac mesenchymal stromal cells and heart section immunofluorescence
Cells were fixed with 4\% paraformaldehyde, paraffin was removed from embedded sections and antigen unmasking was performed. Primary and secondary antibodies staining was carried out in 2\% serum.\textsuperscript{31}

Statistical analysis
Descriptive variables are reported as mean \pm standard deviation. Results were analysed by two-tailed Student’s t-test, Pearson’s correlation test or Fisher’s exact test. Benjamini Hochberg correction was applied when appropriate. Results were considered statistically significant at $P < 0.05$.

Results
Cells differentiating into adipocytes in arrhythmogenic cardiomyopathy human hearts are of mesenchymal origin
To understand the cellular origin of excess adipocytes in ACM hearts, we performed double staining with PLIN1 antibody and mesenchymal markers CD29 and CD105 on serial slices of three ACM explanted hearts (H1–3, Table 1; Figure 1) and three autopsyed control hearts (see Supplementary material online, Table S1). PLIN1 is a protein, specifically expressed in adipocytes,\textsuperscript{32} surrounding lipid droplets. We observed contour staining of large lipid drops of mature adipocytes, as well as small droplets which eventually fuse during maturation of immature adipocytes. We found that all of these differentiating cells express the mesenchymal markers CD29 and CD105 (Figure 2A). We did not detect PLIN1 staining in cells expressing \(\alpha\)-sarcomeric actin (\(\alpha\)SARC; Figure 2B), which suggests that mature cardiomyocytes are not the source of adipogenesis. Further, since the involvement of cardiac progenitor cells was suggested,\textsuperscript{20} we looked for immature adipocytes expressing the c-KIT marker. We detected occasional PLIN1 and c-KIT

Figure 1  Characteristics of an arrhythmogenic cardiomyopathy patient (H1) who underwent heart transplant. (A) Twelve-lead electrocardiogram of a patient with an advanced form of arrhythmogenic cardiomyopathy, recorded 2 years before heart transplant, characterized by sinus rhythm, complete right bundle branch block, ST-T wave abnormalities in leads II, III, aVF, V1–V4 where an e wave can also be identified. Frequent monomorphic premature ventricular beats originating from the inferior right ventricular wall are recorded. (B) Explanted heart of a late-stage arrhythmogenic cardiomyopathy patient, with biventricular involvement. Severe fibro-fatty infiltration is visible in the right ventricle, which is dilated with thin walls. A high-voltage implantable cardioverter defibrillator lead is visible in the right ventricular chamber. (C) Histological preparation of right ventricle tissue sample of the explanted heart (haematoxylin and eosin staining). The scale bar indicates 100 \(\mu\)m.

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double-positive differentiating cells (see Supplementary material online, Figure S1). CD29 and CD105-positive differentiating cells were also found in left ventricle (LV) samples of patients H1 and H2, showing biventricular ACM, whereas only few cells were observed in control hearts (data not shown). Moreover, to verify that pre-adipocytes are of mesenchymal origin even at early phases of the disease, we obtained paraffin sections (FFPE) of biopsies from three patients with mild forms of ACM (B1–3, Table 1). We found fewer pre-adipocytes than in explanted hearts; however, all stained positive for mesenchymal markers (see Supplementary material online, Figure S2A and B). In addition, we did not detect any PLIN1-positive cells co-staining for c-KIT (see Supplementary material online, Figure S2C).

**Cardiac mesenchymal stromal cells can be obtained from arrhythmogenic cardiomyopathy hearts and studied in vitro**

To verify whether the MSC cardiac population may represent the source of excess adipocytes in ACM hearts, C-MSC were isolated from right ventricular endomyocardial biopsy samples from six ACM patients (B4–9, Table 1) and from five control subjects’ right ventricular autopsy samples (see Supplementary material online, Table S1). The biopsy procedure was performed for diagnostic purpose (Figure 3E and F) in patients with electrocardiogram (ECG; Figure 3A) and cardiac magnetic resonance (CMR; Figure 3B and Supplementary material online, Videos A and B) findings suggestive of ACM. Biopptic samples for C-MSC isolation (Figure 3D) were acquired in the area adjacent to the electro-anatomical scars (Figure 3C), without evidence of adipose substitution.

NON-ACM and ACM C-MSC were selected and characterized for morphology and expression of mesenchymal antigens (see Supplementary material online, Figure S3 and Table S2).

**Cardiac mesenchymal stromal cells express desmosomal genes and proteins**

Cardiomyocytes or their progenitors have been previously studied in ACM as the only cardiac cell types expressing desmosome genes. As shown by gene expression analysis in Figure 4A, we demonstrated that desmosomal genes PKP2, JUP, DSG2, DSP, and DSC2 are detectable in isolated C-MSC, and expressed at similar levels in ACM and control cells. Whole ventricle tissue samples were examined as positive controls. This result raised the possibility that C-MSC may be directly affected by ACM-causing mutations. Western blot analysis confirmed that PKP2, PG, DSP, and DSC2 proteins were expressed in ACM and control C-MSC. Plakophilin2 expression was lower in ACM C-MSC (Figure 4B).

**Arrhythmogenic cardiomyopathy cardiac mesenchymal stromal cells show more propensity than controls to differentiate into adipocytes**

When cultured in adipogenic medium (72 h and 1 week), ACM C-MSC accumulated lipids earlier and in significantly higher amounts than NON-ACM control cells (Figure 5). Indeed, after 72 h in adipogenic medium, lipid content, quantified by ORO staining, showed statistically different values between NON-ACM and ACM cells.
This difference persisted after 1 week in adipogenic medium (Figure 5B; 60.83 ± 16.67 vs. 137.40 ± 28.11 a.u., P = 0.001).

The expression of genes involved in adipogenesis or specific for adipocytes was then examined. We found that the enhanced expression of PPARγ, ADIPOQ, FABP4, and PLIN1 after 72 h in adipogenic medium was significantly more pronounced in ACM than in NON-ACM C-MSC (Figure 6A). This result was confirmed by western blot for PPARγ, FABP4, and PLIN1 (Figure 6C). As shown in Figure 6B, a positive correlation exists between ADIPOQ and PLIN1 expression and lipid accumulation analysed by ORO staining (r = 0.874 and r = 0.884, respectively).

In contrast, the anti-adipogenic gene CTGF and the cell-cycle regulator CCND1 were expressed at significantly lower levels in ACM samples than in NON-ACM controls (Figure 6A).

To test the involvement of the WNT pathway, we treated ACM C-MSC with 6-bromoindirubin-3′-oxime (BIO), a drug that inhibits GSK3β. C-MSC cultured with BIO showed a partial (31.25%) reduction in accumulated lipid droplets (P = 0.002) and a 92.24% reduction in ADIPOQ expression (P = 0.045; see Supplementary material online, Figure S4). Notably, PG showed a preferential nuclear localization in ACM C-MSC and a predominant localization in the cytoplasm and at cell–cell junctions in NON-ACM C-MSC (see Supplementary material online, Figure S5).

Lipid accumulation and transcriptional rearrangements in arrhythmogenic cardiomyopathy cardiac mesenchymal stromal cells are dependent on PKP2 deficiency

In order to establish whether the diminished expression of PKP2 plays a causal role in lipid accumulation, ACM C-MSC were transfected either with a plasmid encoding PKP2, or with an empty control vector. PKP2 overexpression was confirmed both at gene and protein levels (Figure 7A). After 72 h in adipogenic medium, cells were examined.
for lipid accumulation and expression of adipogenic genes. Oil Red O staining showed a significant decrease in the number of PKP2-overexpressing cells containing lipid droplets when compared with mock-transfected controls (Figure 7C; 21.33% ± 4.65 vs. 32.57% ± 4.71, P = 0.047). Moreover, PKP2-overexpressing C-MSC showed a 98.10% and a 29.80% decrease in ADIPOQ (P = 0.034) and PPARγ (P = 0.032) expression, respectively (Figure 7B). Finally, we confirmed that lipid accumulation is dependent on PKP2 deficiency by using a specific siRNA during adipogenic induction. Indeed, silencing of PKP2 (see Supplementary material online, Figure S6A) was accompanied by a significant increase in lipid accumulation (15.87 ± 6.99 vs. 38.66 ± 16.91; P = 0.009; see Supplementary material online, Figure S6B).

**Arrhythmogenic cardiomyopathy cardiac mesenchymal stromal cells undergo adipogenesis**

To verify that the accumulation of lipid droplets is associated with C-MSC differentiation to adipocytes and not to mere lipogenesis, we analysed the expression of PLIN1 by qRT-PCR, western blot (Figure 6A and C) and immunofluorescence (Figure 8A). Indeed, ACM C-MSC accumulated lipid surrounded by adipocyte-specific PLIN1 protein. ACM C-MSC cultured in adipogenic medium stained with anti-PLIN1 antibody are phenotypically very similar to the adipogenic differentiating cells detected in ACM patients’ hearts (Figures 2 and 8B). Furthermore, ACM C-MSC maintained the mesenchymal marker CD29 during adipogenesis in vitro (see Supplementary material online, Figure S7).

**Discussion**

To date, ACM has been considered a disease confined to contractile cells even if there is no conclusive experimental evidence to support this hypothesis. To our knowledge, this work is the first demonstration that desmosomal genes are expressed in C-MSC, besides cardiomyocytes, and epithelial cells. Cardiomyocytes have been shown to accumulate lipid in different models, but each of them...
exhibit shortcomings. Indeed, induced pluripotent stem cells (iPSC)-derived cardiomyocytes are a promising patient-specific tool to study ACM as they accumulate lipids. Ongoing studies might overcome the lack of terminal differentiation in cardiomyocytes derived from iPSC, and the presence of residual partially reprogrammed cells. Evidence of desmin and vimentin expression in cells differentiating into adipocytes in heart tissue of a suspected ACM patient has been reported; however, these proteins are not expressed exclusively in cardiomyocytes and adipocytes. Another study claimed that neutral lipids accumulated in ACM right ventricular myocardial tissue, despite this, there was no evidence of myocardial cell differentiation into adipocytes. Finally, transgenic murine models of ACM, expressing different disease-causing mutations under the αMHC promoter, showed the accumulation of adipocytes in the heart, but trans-differentiation of cardiomyocytes into adipocytes was not investigated. In human ACM specimens, we found neither PLIN1-positive lipid droplets accumulating in cardiomyocytes nor αSARC staining in immature adipocytes: this rules out a direct trans-differentiation of cardiomyocytes into adipocytes. Nevertheless, we cannot exclude cardiomyocyte lipogenesis, as previously described.

In the present work, we investigated whether the non-contractile cardiac stromal compartment is the cellular source contributing to excess adipocytes in ACM hearts. Cardiac mesenchymal stromal cells from bioptic samples of patients affected and not affected by arrhythmogenic cardiomyopathy, cultured for 72 h and 1 week in adipogenic medium, stained with Oil Red O (ORO; n = 6 vs. n = 4). The scale bar indicates 50 μm. Quantification of the luminance of the 255 red staining is reported on the right: intensity is expressed in arbitrary units.

Figure 5 Cardiac mesenchymal stromal cells obtained from arrhythmogenic cardiomyopathy patients (ACM), cultured in adipogenic medium, accumulate more lipid droplets than control cardiac mesenchymal stromal cells (NON-ACM). Representative images of cardiac mesenchymal stromal cells from bioptic samples of patients affected and not affected by arrhythmogenic cardiomyopathy, cultured for 72 h and 1 week in adipogenic medium, stained with Oil Red O (ORO; n = 6 vs. n = 4). The scale bar indicates 50 μm. Quantification of the luminance of the 255 red staining is reported on the right: intensity is expressed in arbitrary units.
We also found occasional c-KIT positive cells undergoing adipogenic differentiation in ACM patients’ hearts; however, in our opinion, the rates were too low to account for a major role in lipid accumulation. We have previously shown that c-KIT positive cells can express mesenchymal markers, but they represent only the 0.5–1.5% of isolated C-MSC (see Supplementary material online, Figure S1 and Table S2). Therefore, the diffuse, homogeneous adipogenic differentiation of the whole ACM C-MSC population observed in vitro argues against a major direct contribution of cardiac progenitor cells in the development of fibro-adipose substitution in ACM.

Plakoglobin predominant nuclear staining in cultured C-MSC is consistent with previous findings in other cell types, even if this phenomenon is still controversial in vivo. Therefore, the diffuse, homogeneous adipogenic differentiation of the whole ACM C-MSC population observed in vitro argues against a major direct contribution of cardiac progenitor cells in the development of fibro-adipose substitution in ACM.

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Notwithstanding the relevance of substrate insults in generating or worsening the arrhythmic phenotype of ACM, recent reports highlight ACM as a primary electrical cardiac disease. Although we have observed that in growth conditions Cx43 expression levels and cellular localization (see Supplementary material online, Figure S8) are similar in ACM and NON-ACM C-MSC, future studies are needed to establish whether differentiating C-MSC can contribute to modulate cell survival/death, as well as functional properties of cardiomyocytes, and of other cardiac cell types.

In conclusion, the present article reports unprecedented evidence that C-MSC are a source of adipocytes in ACM heart tissue and that ACM-derived primary C-MSC differentiate into adipocytes in vitro.
Study limitations

The variability we observed among cells derived from different patients, both in lipid accumulation and in gene expression, may at least partially correlate with patient clinical presentation and genetic status. Though limited by the small sample size, an analysis of C-MSC ORO staining intensities stratified by genotype indicated that cells from PKP2 mutation carriers have a higher predisposition to lipid accumulation than C-MSC from patients without mutations in known ACM-related genes ($P = 0.058$). Further, all PKP2 mutation carriers biopsies reached the major diagnostic criterion of substantial fibro-fatty replacement of myocardium. These observations, if validated in studies with larger cohorts, may complete arrhythmic phenotype–genotype correlations reported by Bao and colleagues, who demonstrated that mutation carriers (in particular of PKP2) are subject to spontaneous and induced VT more often than patients without mutations.

We obtained C-MSC only from PKP2 mutation carriers or from patients with no mutations in known ACM-associated genes (Table 1). Therefore, our results need to be tested in patients with other desmosomal or non-desmosomal mutations.
Cardiac mesenchymal stromal cells in arrhythmogenic cardiomyopathy

Cardiac mesenchymal stromal cells in arrhythmogenic cardiomyopathy patients (ACM), cultured in adipogenic medium for 72 h. The scale bar indicates 10 μm. (B) Immunostaining for PLIN1 on FFPE sections of an arrhythmogenic cardiomyopathy (ACM) explanted heart. The scale bar indicates 20 μm. Nuclei are stained with Hoechst 33258.

Not having access to LV bioptic samples of ACM patients, we were unable to explore possible differences in adipogenic propensity between the right and left ventricles.

Supplementary material
Supplementary material is available at European Heart Journal online.

Author contributions

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Conflict of interest: none declared.

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