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Global RNA editing identification and characterization during human pluripotent-to-cardiomyocyte differentiation

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SUMMARY

RNA editing is widely involved in stem cell differentiation and development; however, RNA editing events during human cardiomyocyte differentiation have not yet been characterized and elucidated. Here, we identified genome-wide RNA editing sites and systemically characterized their genomic distribution during four stages of human cardiomyocyte differentiation. It was found that the expression level of ADAR1 affected the global number of adenosine to inosine (A-to-I) editing sites but not the editing degree. Next, we identified 43, 163, 544, and 141 RNA editing sites that contribute to changes in amino acid sequences, variation in alternative splicing, alterations in miRNA-target binding, and changes in gene expression, respectively. Generally, RNA editing showed a stage-specific pattern with 211 stage-shared editing sites. Interestingly, cardiac muscle contraction and heart-disease-related pathways were enriched by cardio-specific editing genes, emphasizing the connection between cardiomyocyte differentiation and heart diseases from the perspective of RNA editing. Finally, it was found that these RNA editing sites are also related to several congenital and noncongenital heart diseases. Together, our study provides a new perspective on cardiomyocyte differentiation and offers more opportunities to understand the mechanisms underlying cell fate determination, which can promote the development of cardiac regenerative medicine and therapies for human heart diseases.

INTRODUCTION

Pluripotent-to-cardiomyocyte differentiation strategies have offered an invaluable model for understanding the detailed mechanisms underlying cell fate determination, which provides opportunities for cardiac regenerative medicine.[1–3] Cardiomyocyte differentiation processes depend on the precise transcriptional and posttranscriptional control of RNA networks, and their disruption may induce congenital heart diseases (CHDs).[4,5] RNA editing is a kind of posttranscriptional modification that changes the sequence of RNA and generates genetic consequences similar to DNA mutations.[6] The most common form of RNA editing in human cells is adenosine to inosine (A-to-I), which is mediated by "adenosine deaminases acting on RNA (ADARs)" editing enzymes.[7] As I is recognized as guanine (G) by translation machines, it is also called the A-to-G variate type. In human cells, three distinct *ADAR* genes have been identified (*ADAR1*, *ADAR2*, and *ADAR3*), of which *ADAR1* (also known as *ADAR*) and *ADAR2* (also known as *ADARB1*) are catalytically activated, whereas *ADAR3* is considered to be catalytically inactive in RNA editing.[8] In addition to A-to-I RNA editing, a small number of other variation types are also identified in human cells, such as T-to-C (U-to-C), G-to-A, and C-to-T (C-to-U). A-to-I editing and C-to-U editing are considered to be canonical RNA editing types, of which A-to-I editing sites are the most prevalent form.[9]

RNA editing is ubiquitously involved in cell development and stem cell differentiation.[10] The deletion of *Adar1* or *Adar2* in mice is embryonically and postnatally lethal, underlying the importance of ADARs and RNA editing events in stem cell differentiation.[11,12] Additionally, disorder of the RNA editing systems will contribute to the alteration of subsequent RNA regulation processes. First, RNA editing of coding sequences in protein-coding genes may change the amino acid translation sequence and influence the function of the corresponding protein.[13] Second, RNA editing may affect the alternative splicing process when editing occurs in splicing sites or splicing regulatory elements.[14] In addition, editing of 3′ untranslated regions (3′ UTRs) may affect the binding of microRNAs (miRNAs).[15] Moreover, editing can also modulate RNA stability and RNA expression.[16,17]

RNA editing in the cardiovascular system has recently captured the interests of researchers.[18] Children with cyanotic CHD showed higher rates of A-to-I RNA editing than children with acyanotic CHD.[19]

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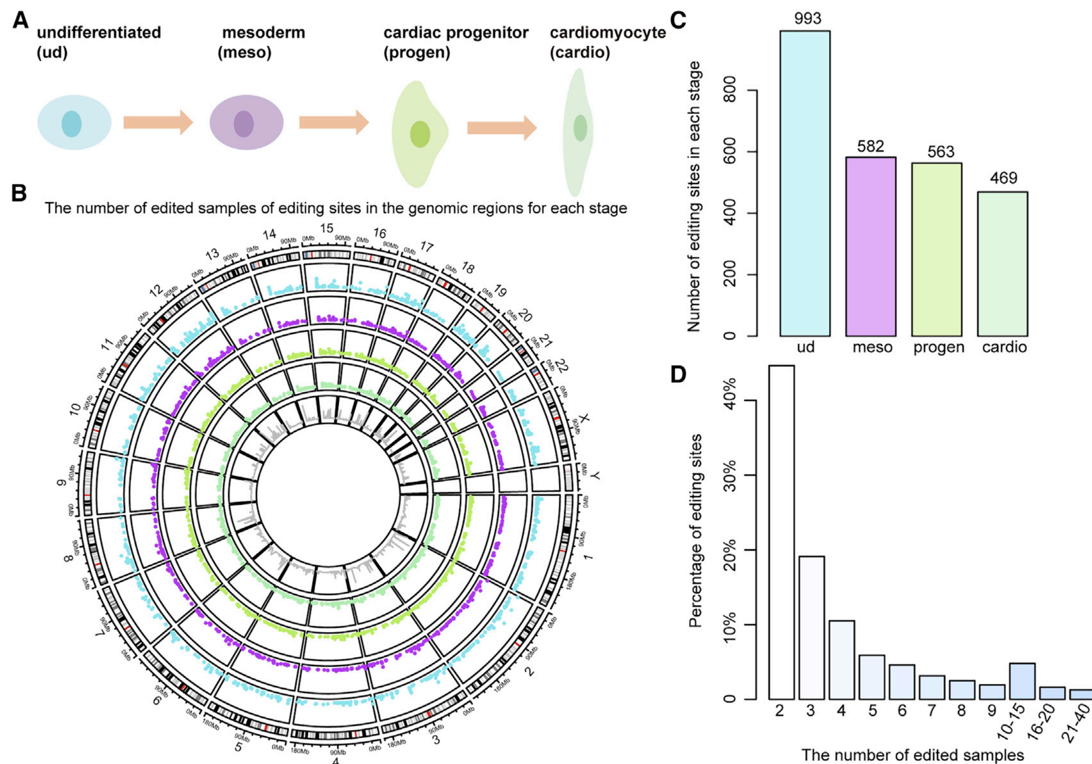

Figure 1. Global distribution of RNA editing sites during cardiomyocyte differentiation

(A) Four differentiation stages were analyzed during human cardiomyocyte differentiation. (B) The chromosome distribution of identified RNA editing sites in each cardiomyocyte differentiation stage. Each region along the circle represents one of the 24 chromosomes. Each circuit denotes a differentiation stage. The circuits represent editing sites identified in undifferentiated (Ud; day 0), mesoderm (Meso; days 2 to 3), cardiac progenitor (Progen; day 5), definitive cardiomyocyte (Cardio; day 14), and total editing sites in all stages from the outer to the inner. (C) The number of RNA editing sites identified in each cardiomyocyte differentiation stage. (D) The distribution of editing percentages across different intervals of samples.

RNA editing events have also been characterized in human atherosclerotic vascular diseases, and cathepsin S (*CTSS*) is highly edited and encodes a cysteine protease associated with angiogenesis and atherosclerosis.[20] Interestingly, in newt hearts, whose cardiomyocytes can be regenerated upon injury, the expression level of *Adar1* mRNA was increased during the first three weeks of heart regeneration, suggesting that RNA editing might be necessary for cardiac regeneration in newts. In addition, the A-to-I RNA editing enzyme Adar1 is essential for normal embryonic cardiomyocyte survival and proliferation.[21] However, no study has comprehensively characterized RNA editing events in human cardiomyocyte development. Moreover, the consequence of RNA editing in cardiomyocyte development remains largely unknown. The understanding of cardiomyocyte development is essential for the study of heart regeneration and repair and necessary for the study of CHDs and even other heart diseases.

In this study, we systemically elucidated RNA editing during four stages of cardiomyocyte differentiation. The expression of *ADAR1* was analyzed, and the effect of RNA editing events on amino acid sequences, alternative splicing, miRNA-target regulation, and gene expression was also dissected during cardiomyocyte development. Stage-specific and stage-shared editing sites were identified and investigated. Finally, we found that RNA editing of cardiomyocyte development is also related to congenital and non-CHDs. Our research is of great significance, which provides new insight into RNA editing during cardiomyocyte development and offers important opportunities for cardiac regenerative medicine and new therapies for heart diseases.

RESULTS

Genome-wide identification of RNA editing sites during human cardiomyocyte differentiation

Human cardiomyocyte differentiation is closely related to heart regenerative medicine. However, RNA editing events during human cardiomyocyte differentiation are largely unknown. Here, we downloaded 71 human cardiomyocyte differentiation samples for 4 cardiomyocyte differentiation stages: undifferentiated (Ud; day 0), mesoderm (Meso; days 2 to 3), cardiac progenitor (Progen; day 5), and definitive cardiomyocytes (Cardio; day 14, Figure 1A). A multistep method was used to gradually identify RNA editing sites by integrating RNA sequencing (RNA-seq) datasets and single-nucleotide polymorphism (SNP) information (Figure S1; details can be seen in

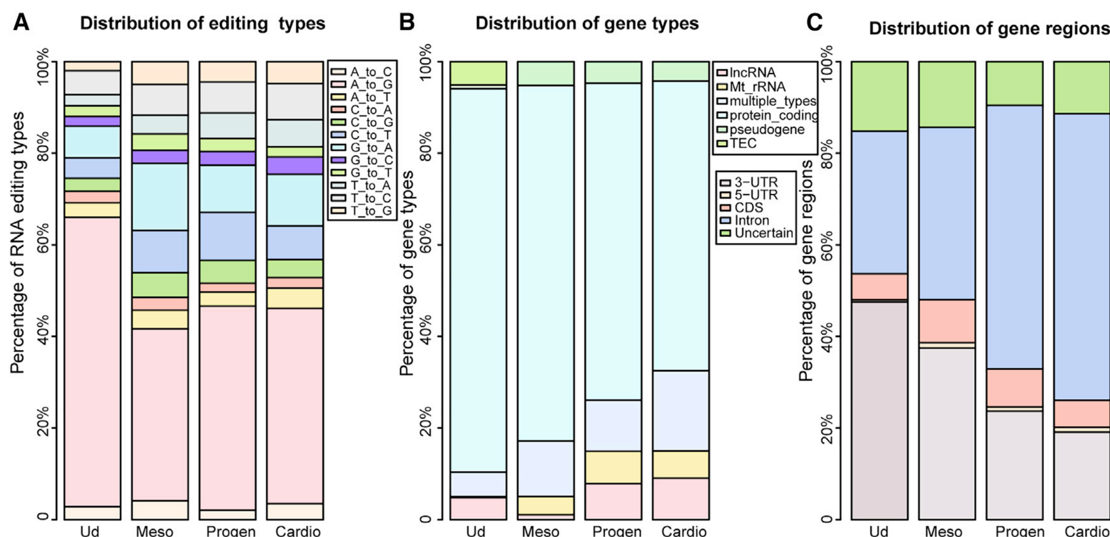

Figure 2. Properties of RNA editing sites during cardiomyocyte differentiation

(A) The distribution of RNA editing variation types across each cardiomyocyte differentiation stage. (B) The distribution of gene types of RNA editing sites across each stage. (C) The distribution of genomic annotations of RNA editing sites across each stage.

Method details). Briefly, we used bwa-mem, an algorithm for Burrows-Wheeler Alignment tool (BWA) to align RNA-seq FASTQ files to the human reference genome (GRCh38) and identified the nucleotide variation files by the Genome Analysis Toolkit (GATK4).[22] After 4 steps of filtering, we obtained 1,561 RNA editing sites across 61 human cardiomyocyte differentiation samples for further analysis with at least 10 coverage reads (Figures 1A and 1B). If we set a looser threshold of minimum coverage reads to 5, a total of 6,563 RNA editing sites were identified during cardiomyocyte differentiation (Figure S2A). Generally, editing events preferred to appear more frequently during the Ud stage (Figure 1C; Figure S2B), which may be due to the larger number of samples for this stage (19 Ud, 16 Meso, 14 Progen, and 12 Cardio samples for each stage). A large number of RNA editing sites were identified in a small number of samples, and approximately half of the editing sites were edited in at least 3 cardiomyocyte differentiation samples (Figure 1D; Figure S2C).

Global characterization of RNA editing sites during human cardiomyocyte differentiation

Previous studies have proven that A-to-I RNA editing is the most common variation type.[9,23] Here, we also found that the A-to-I(G) variant type accounted for most of the RNA variants in each human cardiomyocyte differentiation stage, especially in the Ud (Figure 2A; Figure S3A). Next, we used *BEDTools* to annotate these RNA editing sites to gene types, and it was revealed that most of the editing sites are located at protein-coding genes across 4 cardiomyocyte differentiation stages (Figure 2B; Figure S3B). Moderate RNAs edited at the overlap regions of multiple gene types for 4 stages. Interestingly, there tended to be more editing sites located at long noncoding RNA (lncRNA) regions during the late stage of human cardiomyocyte development in the Progen and Cardio stages (Figure 2B; Figure S3B). Consistent with a previous study,[23,24] most editing sites were located in noncoding regions, such as introns and 3′ UTRs (Figure 2C; Figure S3C). In addition, we found that the percentage of RNA editing sites in the 3′ UTRs decreased while it increased in introns during pluripotent-to-cardiomyocyte differentiation (Figure 2C; Figure S3C).

The expression of *ADAR1* affects the global number of A-to-I editing sites but not the editing degree

A-to-I RNA editing is the most common RNA editing event and is mediated by editing enzymes, including ADAR1 and ADAR2. Here, we focused on these A-to-I RNA editing sites and found high overlap with the RADAR database[24] (79.65%, 642/806 and 78.04%, 2,664/3,414 for coverage read thresholds of 10 or 5, respectively; Figures S4A and S4B), which suggested that the editing sites we identified here are reliable. A total of 465 A-to-I RNA editing sites were edited in at least 3 samples, which were further analyzed to identify ADAR1- or ADAR2-related sites. Here, by Wilcox rank-sum test, 265 and 49 A-to-I RNA editing sites were regulated by ADAR1 and ADAR2, respectively, with a significant difference in *ADAR* mRNA expression between edited samples and samples that did not edit ($p < 0.05$). After Benjamini-Hochberg correction, 208 A-to-I RNA editing sites were related to the RNA expression of *ADAR1,* with no one significantly related to *ADAR2* (adjusted $p < 0.05$; Table 1). The top 3 most significant RNA editing sites are shown in Figure 3A, including chr12;132721380;A;G, which is located at the gene region of *PGAM5*. Importantly, *Pgam5* was reported to drive cardiomyocyte necroptosis by imposing mitochondrial quality control in cardiac ischemia-reperfusion injury in mice and rats.[25,26] Another top 2 A-to-I editing sites were located at the overlap region among *SNHG14*, *SNURF*, and *SNRPN*. Moreover, previous studies reported

Table 1. Several A-to-I RNA editing sites are related to the expression of ADAR1/ADAR2

Number of editing sites	Total A-to-I editing sites	A-to-I editing in ≥3 samples	ADAR1/ADAR2 related A-to-I sites ($p < 0.05$)	ADAR1/ADAR2 related A-to-I sites (FDR < 0.05)
Coverage reads ≥ 10	806	465	ADAR1: 265	ADAR1: 208
			ADAR2: 49	ADAR2: 0
Coverage reads ≥ 5	3,414	1,718	ADAR1: 688	ADAR1: 344
			ADAR2: 191	ADAR2: 0

The p value was measured by the Wilcoxon rank-sum test. FDR denotes false discovery rate, which was measured by Benjamini-Hochberg correction of the p value.

that *SNHG14* and *SNRPN* are involved in the pathogenesis of cardiac hypertrophy and CHD, respectively.[27,28] These results indicated that A-to-I editing events during cardiomyocyte differentiation may also be related to cardiovascular diseases.

Next, we checked the expression of *ADAR1* in 4 stages of human cardiomyocyte differentiation and found that the Ud stage showed significantly higher *ADAR1* expression than the other 3 stages, with the lowest *ADAR1* expression in Cardio stage (Figure 3B). As ADAR1 mediated the A-to-I RNA editing events, the editing degree of A-to-I editing was measured in 4 cardiomyocyte differentiation stages. Interestingly, the global A-to-I editing degree across 4 cardiomyocyte differentiation stages was inconsistent with the expression pattern of *ADAR1*, which was higher in the Cardio stage, while no significant difference among the other 3 stages (Figure 3C). Similar phenomena were seen at the minimum coverage read threshold of 5, which showed no significant difference in editing degree among all 4 differentiation stages (Figure S5A). We further calculated the number of A-to-I RNA editing sites in each sample across 4 cardiomyocyte differentiation stages and found that there was a significantly larger number of A-to-I RNA editing sites in the Ud stage than in the other 3 stages (Figure 3D; Figure S5B). Moreover, the expression level of *ADAR1* was positively correlated with the number of A-to-I RNA editing sites (Pearson correlation coefficient = 0.82; $p < 0.0001$; Figure 3E; Figure S5C). As another ADAR enzyme, the expression level of *ADAR2* was much lower than that of *ADAR1*, and the expression pattern among the 4 differentiation stages was not similar to the editing degree, which showed higher expression at the late stage of differentiation (Figure S6A). The expression of *ADAR2* was not correlated with the number of A-to-I editing sites (Figures S6B and S6C). Taken together, we found that the expression level of *ADAR1* is significantly correlated with the global number of A-to-I editing sites but not the A-to-I editing degree.

The effect of RNA editing on amino acid sequences, alternative splicing, miRNA-target regulation, and gene expression during human cardiomyocyte differentiation

RNA editing is similar to DNA mutation and can lead to a series of functional consequences, such as amino acid sequence changes, alternative splicing variations, and RNA regulation alterations, which have been investigated in human diseases.[29] However, the functional effect of RNA editing in the cardiomyocyte differentiation stage has not yet been clarified. Here, we revealed that 43, 163, 544, and 141 editing sites may lead to amino acid sequence changes, alternative splicing variations, miRNA-target regulation, and gene expression alterations, respectively (Figure 4A). By using *ANNOVA* software, we annotated the editing sites to study their possible effects on amino acid changes and alternative splicing. Here, we found that 43 editing sites can lead to amino acid sequence changes (42 non-synonymous SNVs and 1 stop-loss), which is shown in Figure 4A, with 35 editing sites as synonymous SNVs (Figure 4B). Most editing sites that affect alternative splicing were located at intron splicing regions (Figure 4C; 95.09%, 155/163). Considering the effect of RNA editing on miRNA-target relationships, it was revealed that just a few editing sites influence a large number of miRNA-target bindings (Figure 4D). The top 12 RNA editing sites that influenced at least 35 miRNA-target regulations are shown in Table 2, of which 10 were A-to-I RNA editing variates. Most of the RNA editing sites were located in the noncoding regions of protein-coding genes (Figures 2B and 2C), and here, we found that 141 RNA editing sites had an effect on the expression of the corresponding genes. Interestingly, almost all of the editing events led to higher expression of the corresponding genes when edited (99.29%, 140/141), with only one exception (Figure 4E; Figure S7). The top 6 editing sites with the highest fold change in the expression of the corresponding genes between edited samples and samples that were not edited are shown in Figure S7, and these sites included 4 A-to-I editing sites of *MYL3* and 2 other sites located on *TECRL* and *SLC7A5*. Importantly, *MYL3* is a biomarker of cardiac necrosis,[30] and rare variation in *TECRL* is closely associated with life-threatening arrhythmias and a high risk of sudden cardiac death.[31,32] In general, 77 RNA editing sites influenced at least 2 functional events that we studied, as shown in the right panel of Figure 4A, including editing sites located at the abovementioned *PGAM5*, which is associated with heart diseases. To investigate whether the editing degree is associated with the corresponding gene's expression, we also performed correlation analysis and identified 196 editing sites whose editing degrees are significantly correlated with the corresponding gene's expression, of which 133 were also identified in Figure 4E whose gene's expression was also significantly different between edited samples and samples that did not edit (94.33%, 133/141).

By using the minimum coverage read threshold of 5, 115, 848, 1,628, and 336 editing sites had an effect on amino acid sequences, alternative splicing, miRNA-target binding, and gene expression, respectively, and similar results are shown in Figures S8A–S8E.

Stage-specific and stage-shared RNA editing events during human cardiomyocyte differentiation

Different genes play different roles during human cell development. Therefore, we hypothesized that editing events may also function differently among the 4 cardiomyocyte differentiation stages. Here, we compared RNA editing sites among 4 human cardiomyocyte differentiation stages and identified 993, 582, 563, and 469 editing sites in the Ud, Meso, Progen, and Cardio stages, respectively (Figures 1C

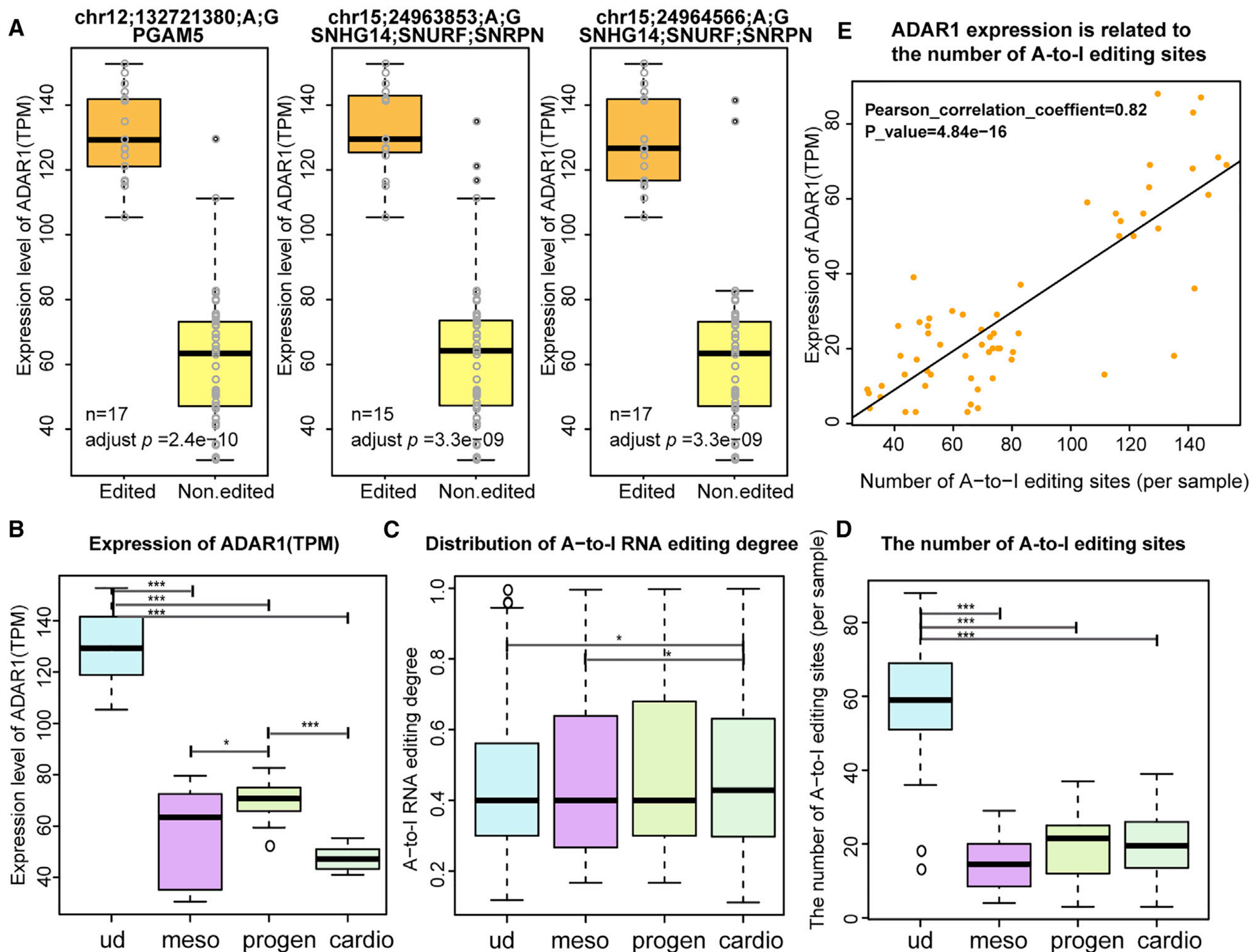

Figure 3. The expression of level of ADAR1 is correlated with the global number of A-to-I editing sites

(A) Example of the top 3 editing sites that were most statistically significant. The expression levels of ADAR1 were significantly different between edited samples and samples that did not edit for these 3 editing sites (Wilcoxon rank-sum test with Benjamini-Hochberg correction). (B) Expression of ADAR1 across 4 cardiomyocyte differentiation stages. (C) Distribution of A-to-I RNA editing degree across 4 cardiomyocyte differentiation stages. (D) The number of A-to-I editing sites across 4 cardiomyocyte differentiation stages. In (B)–(D), the p value was measured by Wilcoxon rank-sum test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (E) ADAR1 expression is related to the number of A-to-I editing sites. The p value and correlation coefficient were measured by Pearson correlation test.

and 5A). Of these, 774 were stage-specific editing sites, including 470 Ud-specific, 56 Meso-specific, 120 Progen-specific, and 128 Cardio-specific editing sites, with 211 stage-shared editing sites that appeared in at least 3 differentiation stages (Figure 5A).

After mapping stage-specific editing sites to genes, we found that most of the different stage-specific editing sites were located in the same gene regions, with less than 30% of the editing sites located in unique gene regions (234/774; Figure 5B). Interestingly, for these approximately 70% gene-shared stage-specific editing sites, we found that they tended to be identified in the same differentiation stages (88.33%, 477/540) with a few exceptions that were identified in different stages (11.67%, 63/540; Figure 5C; Table 3), which is similar to the phenomenon observed in hepatocellular carcinoma.[29] Among these stage-specific and stage-shared editing sites, 171 had at least 1 functional consequence, such as amino acid sequence changes, alternative splicing variations, miRNA-target regulation, and gene expression alterations, as shown in Figure 4. 11 Ud-specific editing sites were located in regions of NANOG (Table 3), of which 6 have a potential effect on miRNA-target changes and expression changes in NANOG (Figure 4A). Importantly, Nanog can maintain the cell self-renewal properties of mouse embryonic stem cells (ESCs) and is recognized as a critical pluripotency gene in stem cell regulation.[33] For Cardio-specific editing sites, we found 8 sites edited at genomic regions of MYL3 (Table 3), of which 6 A-to-I editing sites were related to high expression levels of MYL3 in edited samples compared with nonedited samples (Figure 4E). Moreover, MYL3 is a protein involved in muscle contraction and associated with cardiac necrosis.[30]

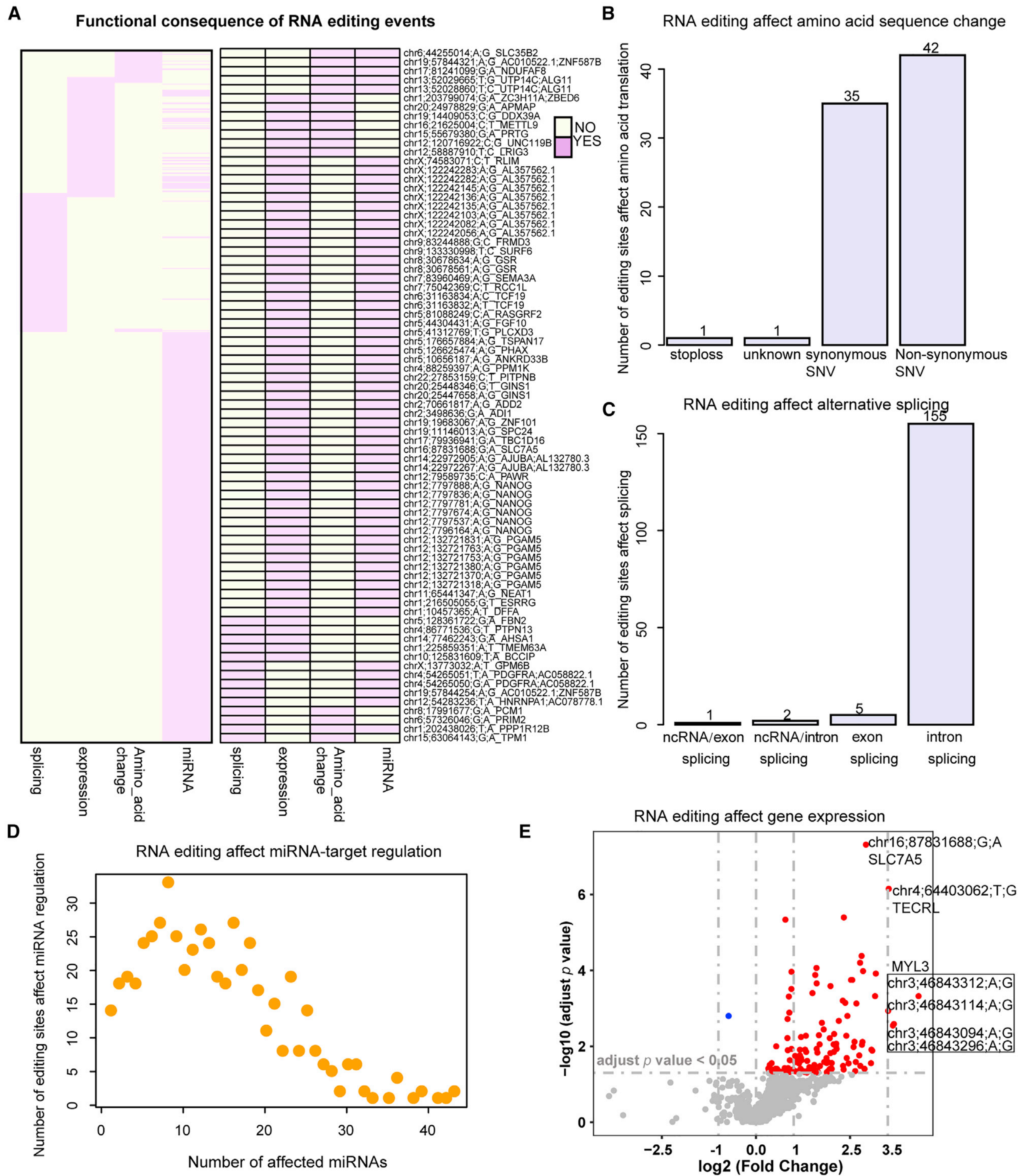

Figure 4. Downstream possible functional consequence of RNA editing sites during cardiomyocyte differentiation

(A) The functional consequence of RNA editing, which affects amino acid translation, alternative splicing, miRNA-target regulation, and corresponding gene expression. The right panel shows editing sites with at least 2 functional consequences. (B) The number of RNA editing sites that affect the change in amino acid sequence. (C) The number of RNA editing sites which affect alternative splicing. (D) The number of RNA editing sites that affect miRNA-target regulation. (E) Volcano plot of the expression change between edited samples and nonedited samples for a given editing site. The p value was measured by Wilcoxon rank-sum test and adjusted by Benjamini-Hochberg.

Table 2. The top 12 RNA editing sites that influence at least 35 miRNA-target regulations are shown.

RNA editing sites	Genes	Number of affected miRNA-target regulations
chr19;57305637;A;G	*AC005261.1*	43
chr17;75889858;A;G	*TRIM65*	43
chr6;149725359;A;G	*NUP43*	42
chr17;44802946;A;G	*GJC1*	41
chr12;132721535;A;G	*PGAM5*	39
chr11;116778835;T;C	*ZPR1*	39
chr14;74736154;A;G	*FCF1*	38
chr8;30679086;A;G	*GSR*	36
chr2;74493781;C;T	*TTC31*	36
chr19;52878521;A;G	*ZNF320*	36
chr12;132721669;A;G	*PGAM5*	36
chr4;188004400;A;G	*ZFP42*	35

BMPER, also known as CV2, is a bone morphogenetic protein (BMP)-binding protein that modulates the activity of several BMPs, which are essential in angiogenesis and cardiomyogenesis.[34] Here, we found that BMPER is a Progen-specific editing gene that has 8 editing sites specifically identified in the Progen stage (Table 3), of which 2 sites whose editing is related to expression changes in BMPER (chr7;33949687;T;C, chr7;33959595;A;G). Functional enrichment analysis was performed by the genes of these stage-specific editing sites and revealed that Cardio-specific editing sites are were enriched in heart-related biological processes (BPs), such as "regulation of muscle contraction," "cardiac muscle tissue development," and "cell communication involved in cardiac conduction" (Figure 5D). The Progen-specific editing sites are involved in morphogenesis and myofile- and neuron-related processes, such as "cellular component," "myofibril assembly," and "neuron projection morphogenesis." RNA processing and metabolism-related processes were enriched in Meso-specific editing sites, while ribosome biogenesis and cell-cycle related processes were involved by in Ud-specific editing sites. For the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis, we found that pathways of cardiac-associated diseases were enriched by genes of Cardio-specific editing sites, such as "hypertrophic cardiomyopathy (HCM)," "arrhythmogenic right ventricular cardiomyopathy (ARVC)," and "dilated cardiomyopathy (DCM)" (Figure 5E).

For the genes of stage-shared RNA editing sites, we found that "RNA processing," "spliceosome," "blastocyst development," and "cell communication involved in cardiac conduction" were enriched (Figures S9A and S9B). Among these 211 stage-shared RNA editing sites, we measured the editing degree difference among 4 differentiation stages and identified that just 1 mitochondrial A-to-I editing site was significantly differentially edited, which showed that the editing degree was higher when the stage of development was higher and located at *MT*−*RNR2* (Figure S9C). Moreover, *MT-RNR2* mutation in induced pluripotent stem cell (iPSC)-derived cardiomyocytes contributes to HCM with mitochondrial dysfunction.[35,36] This underlines that the editing of *MT-RNR2* may also change mitochondrial function during cardiomyocyte differentiation stages.

Although more stage-specific and stage-shared RNA editing sites were identified at a lower threshold of minimum coverage read depth (Figure S10A), it was also revealed that different stage-specific editing sites preferred to be at same gene regions (Figure S10B), and gene-shared stage-specific editing sites tended to be identified in the same differentiation stages (Figure S10C). Moreover, similar gene sets were enriched for each stage-specific editing gene, such as Cardio-specific editing sites enriched in gene sets related to heart diseases and cardiac muscle development (Figures S10D and S10E).

Several congenital and non-CHDs are related to editing sites during human cardiomyocyte differentiation

During cardiomyocyte differentiation, RNA editing genes were enriched in gene sets related to cardiac cell development and heart diseases, especially genes of Cardio-specific RNA editing sites (Figures 5D and 5E; Figures S10D and S10E). Here, we identified DNA/RNA variants from patients with human CHDs, including single ventricular diseases (SVDs), tetralogy of fallots (TOFs), and control samples. A total of 4,462 SVD-related and 2,541 TOF-related DNA/RNA variants were identified as compared with control samples (Figure 6A; Figure S11A). This result suggested that there is a high overlap of DNA/RNA variance between these two CHDs and that a few overlap with the RNA editing sites we identified during the cardiomyocyte differentiation (Figure 6A; Figure S11A). Underlying RNA editing events during human cardiomyocyte differentiation may have an effect on the normal morphogenesis and function of the heart. Moreover, we found that RNA editing events specific to the Ud stage may be more important for the consequence of SVD (6.38%, 30/470 and 4.63%, 69/1491 for coverage read thresholds of 10 and 5, respectively), followed by stage-shared editing set (4.74%, 10/211 and 4.13%, 43/1042 for coverage read thresholds of 10 and 5, respectively). For TOFs, stage-shared RNA editing events may be more relevant (7.58%, 16/211), followed by Cardio-stage-specific (4.69%, 6/128) and Ud-stage-specific editing sites (3.40%, 16/470; Figure 6B) at a minimum coverage read threshold of 10, which is slightly different from the results obtained by a minimum coverage read threshold of 5, of which the top enriched site was the Meso-specific editing set (5.20%, 13/250; Figure S11B). In addition, a small number of RNA editing sites during human cardiomyocyte differentiation were also identified as DCM-related variant sites (Figure 6C; Figure S11C), and Cardio-specific editing sites were enriched in DCM-related sites (3.91%, 5/128 and 1.57%, 7/445 at coverage read thresholds of 10 and 5, respectively), followed by stage-shared editing sites (1.90%, 4/211 and 1.06%, 11/1042 at coverage read thresholds of 10 and 5, respectively; Figure 6D; Figure S11D), which is consistent with the result that genes of Cardio-specific editing sites were significantly enriched in pathways of cardiac-related processes and heart diseases by using BP and KEGG datasets (Figures 5D and 5E; Figures S10D and S10E). Taken together, it was concluded that several RNA

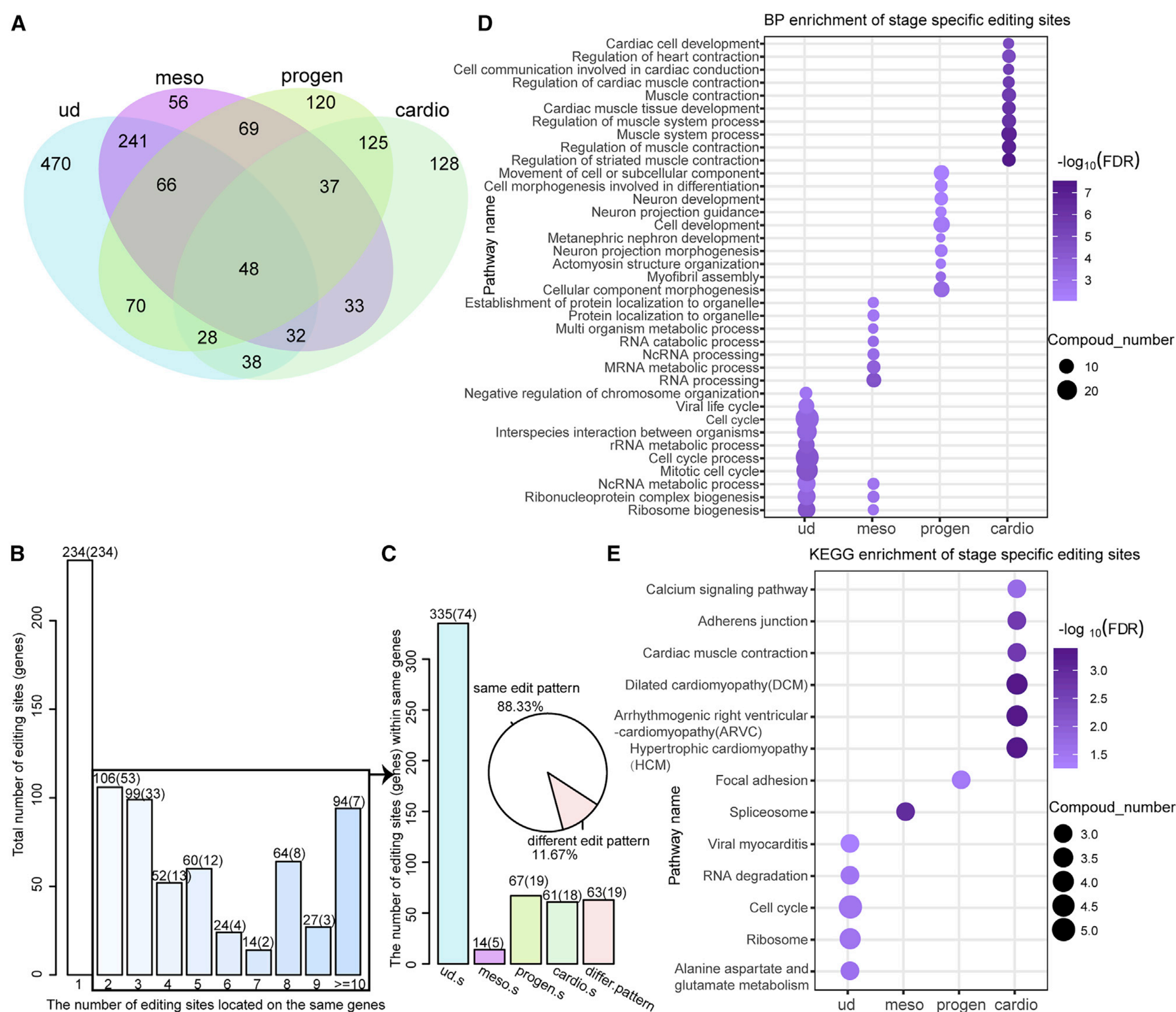

Figure 5. Stage-specific RNA editing events during human cardiomyocyte differentiation

(A) The overlap of RNA editing sites among 4 cardiomyocyte differentiation stages. (B) Number of RNA editing sites located at the same gene regions. The y axis denotes the total number of stage-specific RNA editing sites, and the parentheses show the number of genes involved. The x axis suggests the number of RNA editing sites located at the same gene. (C) Different stage-specific RNA editing sites within the same genes tend to have the same editing patterns. (D and E) Biological process (D) and KEGG (E) enrichment of genes involved in each stage-specific RNA editing site (hypergeometric test with Benjamini-Hochberg correction; adjusted $p < 0.05$). BPs were listed as the top 10 most significantly enriched processes in each stage.

editing sites during human cardiomyocyte differentiation were also connected to human heart diseases, including CHDs and non-CHDs, which may provide opportunities for new therapies for heart disease and deserve further analysis.

DISCUSSION

RNA editing is essential for human embryogenesis in a stage-specific fashion.[37,38] However, RNA editing events are unexplored during the development of the cardiovascular system.[18] Here, we identified RNA editing sites genome-wide during human cardiomyocyte differentiation and manifested the genomic distribution of editing events. Consistent with previous studies, RNA editing sites are found mostly to be A-to-I variant types and are preferentially located on non-protein-coding regions, especially in introns and 3′ UTRs.[23,24] Notably, we found that the top 4 major RNA editing variant types identified in our study were A-to-G, T-to-C, G-to-A, and C-to-T (Figure 2A). A-to-G and C-to-T variants are canonical editing types. T-to-C and G-to-A variants can be understood as possible A-to-I editing and C-to-U editing, respectively, if we consider antisense transcription.[9] Taking this into consideration, the 2 known RNA editing types (A-to-I and

Table 3. Stage-specific RNA editing sites located in the same gene regions tended to be identified in the same differentiation stages.

Gene	Frequency	HCC related sites
SNHG14;SNURF;SNRPN	18	18 Ud-specific
AL357562.1	15	15 Ud-specific
GJC1	15	15 Ud-specific
DFFA	14	14 Ud-specific
ZDHHC20	11	11 Ud-specific
NANOG	11	11 Ud-specific
DANT2	10	7 Cardio-specific; 3 Progen-specific
MAGT1	9	9 Ud-specific
EIF2AK2"	9	9 Ud-specific
BDNF-AS	9	9 Ud-specific
GSR	8	6 Ud-specific; 2 Cardio-specific
BMPER	8	8 Progen-specific
MYL3	8	8 Cardio-specific
DPPA4	8	8 Ud-specific
DNMT3B	8	8 Ud-specific
AC010522.1;ZNF587B	8	8 Ud-specific
PGAM5	8	8 Ud-specific
HELLS	8	8 Ud-specific
FBN2	7	7 Cardio-specific
GINS1	7	7 Ud-specific

Note: This table shows the top 9 genes that were related to at least 9 RNA editing sites.

C-to-U) together accounted for most of the RNA variants during cardiomyocyte differentiation (Table S1), which is consistent with other studies, and A-to-I editing sites showed a disproportionately high percentage.[9,29] In particular, we also confirmed that approximately 79% of the identified A-to-I editing sites have been found in previous studies stored in the RADAR database. These results collectively indicate that the RNA editing sites identified here are credible.

Interestingly, we found that the expression level of *ADAR1* was higher than that of *ADAR2* (Figure 3B; Figure S6A) and the *ADAR2* level was increased in the late stage of differentiation, which is consistent with a previous study showing that ADAR enzymes play distinct roles in myeloid leukemia cells. ADAR1 is present at the basal level while ADAR2 is absent in the Ud cell stage and strongly upregulated at the end of the differentiation process.[39] Therefore, we suggest that ADAR1 plays an important role during human cardiomyocyte differentiation, especially in the early stage. Furthermore, we found that the expression of *ADAR1* is increased in the Ud differentiation stage compared with the other 3 stages, while the editing degree does not show a similar pattern to *ADAR1* expression. This is consistent with previous studies that revealed a lack of direct correlation between editing levels and *ADAR* mRNA expression during mouse and rat brain development.[6,40] Interestingly, we found that more editing sites were identified when *ADAR1* expression was high in the Ud stage, with a high positive correlation between *ADAR1* expression levels and the number of A-to-I RNA editing sites. Moreover, Sagredo et al.[41] also reported a significant correlation between *ADAR1* expression and the number of A-to-I sites identified in breast cancer cell lines. These results suggested that ADAR1 may play important roles in RNA editing events during human cardiomyocyte differentiation, which was consistent with previous studies reporting that ADAR1 is essential for normal embryonic cardiac growth and development, and that cardiomyocyte-specific deletion of ADAR1 causes severe cardiac dysfunction and increased lethality.[21,42]

Our research also has some limitations that need to be further investigated. First, the sequencing depth of RNA-seq datasets during heart development was too low (Figure S12), and approximately 16 million reads were identified in each sample,[43] which led to a small number of RNA editing sites identified here at the minimum coverage read threshold of 10. Considering the low sequencing depth, we also used a looser threshold of coverage read to 5 to obtain more candidate RNA editing sites (Figure S2). The results are similar to those described before (see Supplemental information for details). In the future, sequencing data with higher sequencing depth during cardiomyocyte differentiation should be used to further verify and expand our results. Second, due to the lack of corresponding DNA-seq datasets during fetal heart development, we cannot rule out DNA mutation sites from identified DNA/RNA variance. However, as DNA mutation is a rare event in normal cell development, we ignored this during human cardiomyocyte differentiation. However, DNA mutations are more likely to be involved in heart diseases, especially CHDs. Although we cannot distinguish whether the variance is DNA mutation or RNA editing during heart diseases, we can conclude that several RNA editing sites during cardiomyocyte differentiation are also identified as DNA mutations or RNA editing sites, which are related to heart diseases. In addition, we limited the consequence analysis to the effect of amino acid sequence change, alternative splicing variation, gene expression alteration, and miRNA-target regulation change, whereas the function of RNA-binding proteins may also be changed after RNA editing, and RNA binding proteins play important roles in regulating RNA editing,[44,45] which deserves further analysis. Moreover, the immune regulation and m6A regulators may also play important roles in RNA editing, which also needs to be further investigated.[46,47]

MATERIALS AND METHODS

RNA editing identification during human cardiomyocyte differentiation stages

Paired-end RNA-seq datasets of cardiomyocyte differentiation stages performed on the Illumina HiSeq 2500 platform were downloaded from the Gene Expression Omnibus (GEO) database (GEO: GSE64417; Table S2).[43] In detail, these RNA-seq datasets included 71 samples for 4 cardiomyocyte differentiation stages: Ud (day 0), Meso (day 2), Progen (day 5), and Cardio (day 14).

The method for the identification of RNA editing was described in our previous study[29] with small modifications due to no corresponding DNA mutation information here (Supplementary File S1). Briefly,

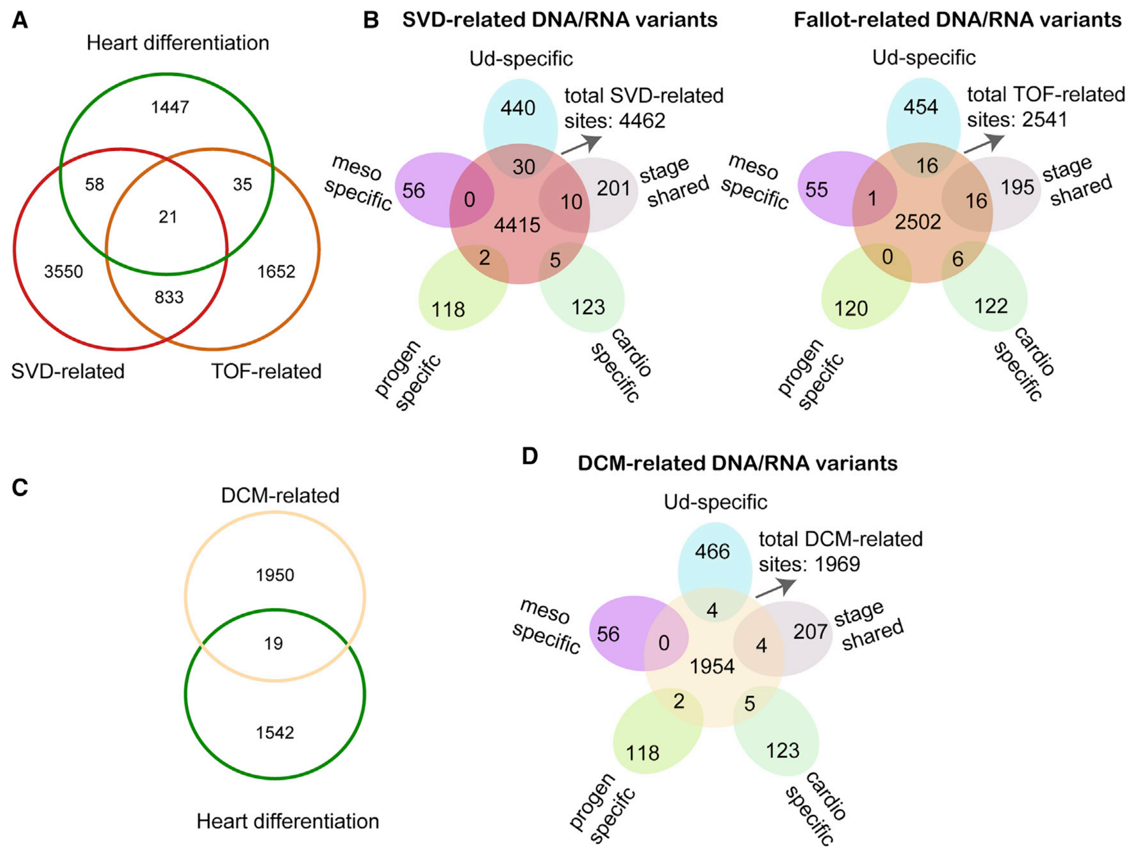

Figure 6. The overlap of RNA editing sites during human cardiomyocyte differentiation and DNA/RNA variance associated with heart diseases
(A) The overlap of RNA editing sites identified in cardiomyocyte differentiation stages and DNA/RNA variance associated with congenital heart diseases. (B) The overlap of stage-specific and stage-shared RNA editing sites during cardiomyocyte differentiation and SVD-related (left panel) or TOF-related (right panel) DNA/RNA variance. (C) The overlap of RNA editing sites identified in cardiomyocyte differentiation stages and DNA/RNA variance associated with DCM. (D) The overlap of stage-specific and stage-shared RNA editing sites during cardiomyocyte differentiation and DCM-related DNA/RNA variance.

the raw FASTQ files were extracted by fastq-dump from the Sequence Read Archive (SRA). FASTQ datasets were then aligned to the human reference genome (GRCh38) by using *bwa-mem* (bwa-0.7.17) and *SAMtools* (samtools-1.5) with default parameters.[48] The human reference genome GRCh38 and the corresponding GTF annotation files were downloaded from GENCODE (https://www.gencodegenes.org/).[49] The nucleotide variation files were identified using uniquely mapped reads by the GATK4 with default parameters.[22] Then, 4 steps of computational filters were performed to identify RNA editing sites among cardiomyocyte differentiation stages: (1) Removing all known SNPs in dbSNP version 137[50] or 1000 Genomes project,[51] and removing insertion or deletion sites to obtain single nucleotide change types. (2) Further filtering to obtain highly confident editing sites: we restricted RNA editing supported by at least 2 reads, with at least 10 coverage reads (a looser threshold of minimum coverage reads to 5 was also analyzed), FisherStrand (FS) $\leq$ 20 and quality by depth (QD) $\geq$ 2. (3) As 100% editing efficiency is thought to be unrealistic,[23] we also removed sites with 100% editing degree. (4) Variants were restricted to be detected in at least 2 samples because editing is unlikely to be rare variants. At a given site, editing degree was defined as the percentage of edited reads among the total mapped reads.[52] (5) We restricted editing sites to 46 human chromosomes and mitochondrial DNA. *BEDtools*[53] was used to map editing sites with a human gene annotation GTF file. If the RNA editing sites were simultaneously mapped to 2 strands or annotated to intergenic regions, these sites were also removed. (6) We harvested 1,561 (6,563 at a minimum coverage read threshold of 5) RNA editing sites from 61 samples for further analysis, with 19 Ud, 16 Meso, 14 Progen, and 12 Cardio samples in each differentiation stage.

DNA/RNA variance identification in human CHDs and human DCM

The paired-end RNA-seq datasets of 10 CHDs, including 5 SVDs and 5 TOFs, and 5 healthy control samples were downloaded from the GEO database (GEO: GSE132401) performing on the Illumina NovaSeq 6000 platform[54] (Table S2). Another paired-end RNA-seq dataset contained 3 human DCM samples and 3 control samples from the GEO database (GEO: GSE162505)[55] (Table S2). The DNA/RNA variances were identified using the same method described in Figure S1,

which was the same as the identification of RNA editing during human cardiomyocyte differentiation mentioned above.

Then, we identified DCM-gain (specific in at least 2 DCM patients with no control samples) and DCM-lost (specific in at least 2 control samples with no DCMs) DNA/RNA variances, which were both defined as DCM-related DNA/RNA variances. Similarly, SVD-related DNA/RNA variances and TOF-related DNA/RNA variances were also identified.

Characterization of RNA editing sites during human cardiomyocyte differentiation

BEDtools was used to map editing sites with a human gene annotation GTF file to obtain the gene-type information of RNA editing sites. If a given RNA editing site was located at the overlap region of 2 gene types, the gene type was referred to as multiple types.

Gene annotation of intron, 3′ UTR, 5′ UTR, and Coding sequence (CDS) regions was obtained from the UCSC table browser (http://genome.ucsc.edu/cgi-bin/hgTables).[56] *BEDtools* was used to map the intron, 3′ UTR, 5′ UTR, and CDS regions with RNA editing sites.

The A-to-I RNA editing sites were regulated by ADAR enzymes

We collected known A-to-I RNA editing sites from the RADAR database,[24] which refers to the human reference genome GRCh37. The A-to-I RNA editing sites for human cardiomyocyte differentiation were lifted over GRCh38 to GRCh37 by the UCSC *liftOver* tool. Then, we compared and measured the overlap of A-to-I RNA editing sites between our study and the RADAR database.[24]

To identify A-to-I RNA editing sites regulated by *ADARs* for a given RNA editing site, we classified heart samples into "edited samples" and "nonedited samples," and then the differential expression of *ADARs* (*ADAR1* and *ADAR2*) was analyzed between these 2 groups by the Wilcoxon rank-sum test (Benjamini-Hochberg correction; adjusted $p < 0.05$).

Gene expression during human cardiomyocyte differentiation

After aligning the FASTQ files to the human reference genome (GRCh38) by *bwa-mem* (bwa-0.7.17) and *SAMtools* (samtools-1.5), gene expression was calculated by *StringTie*[57] with default parameters. Then, we obtained the expression profile of 61 samples and kept the genes with transcripts per kilobase of exon model per million mapped reads (TPM) >0 in at least 10% of heart samples.

Downstream functional consequence analysis for RNA editing sites

1) RNA editing sites to influence amino acid sequences and alternative splicing

ANNOVAR software was used to define whether an editing site can lead to amino acid sequence changes or alternative splicing variations.[58]

2) RNA editing sites to affect miRNA target binding.

For each RNA editing site located at 3′ UTR regions, we used *miRanda*[59] to computationally predict the possibility of a miRNA binding to the RNA regions around editing sites as described by Hwang et al.[9] Briefly, for each RNA editing site, we downloaded RNA sequences for flanking regions of the editing site (50 bp upstream and downstream) from UCSC and prepared 2 types of sequences: the reference sequence and the editing sequence. The mature miRNA sequences were obtained from miRBase.[60] Then, we used *miRanda* (v3.3a; default parameters) to calculate the binding energies between miRNA sequences with 2 types of RNA sequences: reference and edited mRNA sequences, respectively. To obtain more confident miRNA-target regulations, delta G was set to be less than −14 kcal/mol for the free energy of duplex formation. Then, miRNA-target relationships were compared between reference sequences and edited sequences and defined "edited loss" (just appeared in reference but not in edited sequence), "edited gain" (just appeared in edited but not in reference sequence), and "edited change" (at least 14 kcal/mol binding energies differences between reference and edited sequences). The 3 types of editing sites that affect miRNA-target binding were taken together to be considered as having a miRNA-target binding consequence.

3) RNA editing sites to affect corresponding gene expression.

First, we used *BEDtools* to obtain the corresponding gene names of editing sites. To define whether RNA editing can influence the corresponding gene expression, differential expression analysis was performed by the Wilcoxon rank-sum test (Benjamini-Hochberg correction; adjusted $p < 0.05$).

Stage-specific and stage-shared RNA editing sites were identified during human cardiomyocyte differentiation

The RNA editing sites among 4 cardiomyocyte differentiation stages were compared and defined to be stage-specific (identified only in the specific cardiomyocyte differentiation stage) and stage-shared (identified in at least 3 cardiomyocyte differentiation stages).

For each group of RNA editing sites, the corresponding genes of editing sites were used to perform functional enrichment analysis of KEGG and BPs of Gene Ontology (GO) classes by hypergeometric testing (Benjamini-Hochberg correction; adjusted $p < 0.05$). The annotated gene sets were downloaded from the MsigDB database (https://gsea-msigdb.org/gsea/msigdb),[61] including KEGG and BP of GO classes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.10.001.

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AUTHOR CONTRIBUTIONS

J.C. and J.L. designed the study. H.-f.L. and L.-b.Q. collected and processed the sequencing data. J.C., L.W., F.-b.W., and Y.L. analyzed the data. J.C. wrote the article. All authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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