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1 *Article*

2 Polysome-associated lncRNAs during 3 cardiomyogenesis of hESCs

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9

10 **Abstract:** Long non-coding RNAs (lncRNAs) have been found to be involved in many biological
11 processes, including the regulation of cell differentiation, but a complete characterization of lncRNA
12 is still lacking. Additionally, there is evidence that lncRNAs interact with ribosomes, raising
13 questions about their functions in cells. Here, we used a developmentally staged protocol to induce
14 cardiogenic commitment of hESCs and then investigated the differential association of lncRNAs
15 with polysomes. Our results identified lncRNAs in both the ribosome-free and polysome-bound
16 fractions during cardiogenesis and showed a very well-defined temporal lncRNA association with
17 polysomes. Clustering of lncRNAs was performed according to the gene expression patterns during
18 the five timepoints analyzed. In addition, differential lncRNA recruitment to polysomes was
19 observed when comparing the differentially expressed lncRNAs in the ribosome-free and
20 polysome-bound fractions or when calculating the polysome-bound vs ribosome-free ratio. The
21 association of lncRNAs with polysomes could represent an additional cytoplasmic role of lncRNAs,
22 e.g., in translational regulation of mRNA expression.

23 **Keywords:** long non-coding RNA, hESC, cardiomyocyte, RNA-seq
24

25 1. Introduction

26 Long non-coding RNAs (lncRNAs) are non-coding RNAs that are arbitrarily characterized as
27 being longer than 200 bp and are structurally indistinguishable from protein-coding and processed
28 messenger RNAs since they are also 5′ capped and polyadenylated [1,2]. A plethora of lncRNAs have
29 been identified that are associated with many biological processes, such as embryogenesis, the cell
30 cycle, pluripotency, apoptosis and differentiation [1,3]. LncRNAs are more tissue-specific than
31 protein-coding genes; however, they tend to be expressed at low levels [4–6]. LncRNAs can be
32 classified according to their proximity to protein-coding genes as sense, antisense, divergent, intronic
33 or intergenic [7].
34 The mechanisms by which lncRNAs act still need to be determined, but there is robust evidence
35 that they play crucial roles in gene expression regulation at different levels, including chromatin
36 organization, transcriptional regulation and post-transcriptional control [7]. Although there is a lack
37 of conserved lncRNA sequences between species [4,8,9], their secondary structure might actively
38 participate in their function through interactions with proteins and other RNAs [10–12].
39 LncRNAs can also interact with ribosomes and can be found in polysomal complexes [13–16].
40 The association of lncRNAs with polysomes might indicate the coding potential of these transcripts,
41 representing a source of new peptides [17]. On the other hand, ribosome footprints have been found
42 along lncRNAs as well as in previously known non-coding sequences, suggesting pervasive
43 translation in the cell [18]. In addition to the coding potential of lncRNAs, their association with
44 polysomes could represent a role in translational regulation, for instance, by fine-tuning the speed or
45 specificity of the machinery and titrating out ribosomes [1]. In addition, the base pairing capability
46 of lncRNA indicates that they can interact with and regulate specific mRNAs [7,11].

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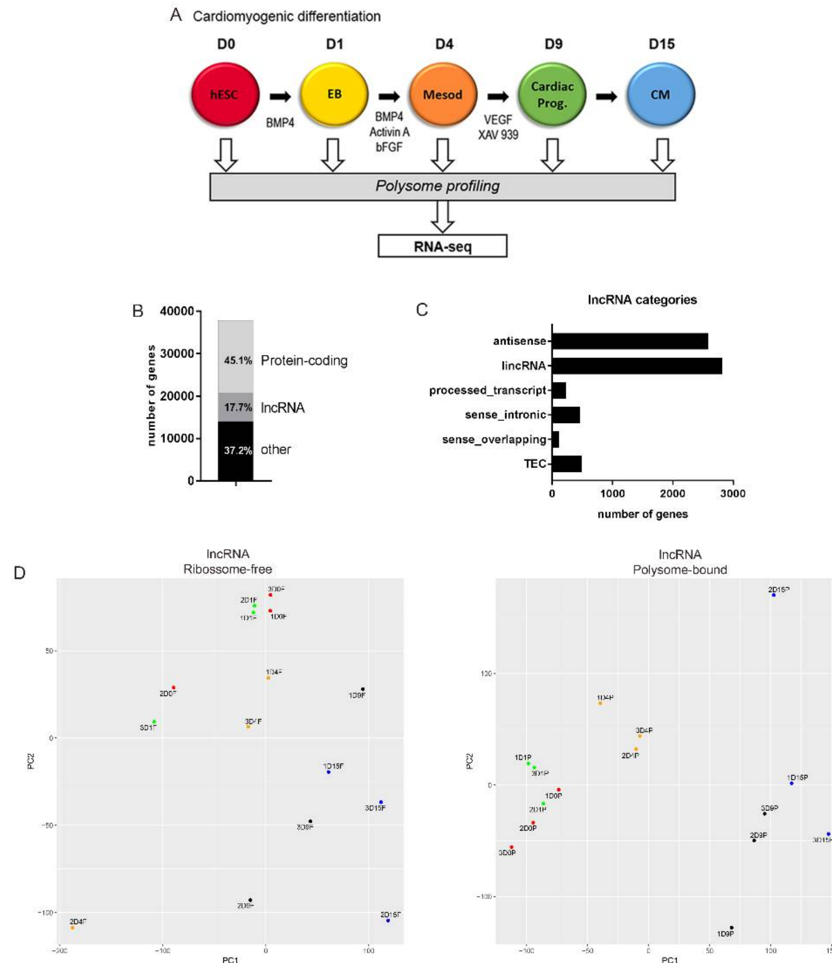
Cell differentiation requires the activation of specific genetic programs, and lncRNA expression has been found to contribute to cell commitment processes (revised by [3]). Analysis of the murine and human stem cell transcriptomes during cardiomyocyte differentiation revealed thousands of differentially expressed lncRNAs [19–23]. These lncRNAs have temporal-specific expression, suggesting that they play a crucial role in cardiac development regulation [19–24]. Identification of new lncRNAs and their tissue specificity and function could reveal that they are a new class of regulatory molecules in cardiac development [25].

Our group has previously used polysome profiling followed by RNA-seq to analyze the expression and polysome association of lncRNAs in undifferentiated human adipose-derived stem cells and committed to differentiation into adipose cells [14]. Here, we used a hESC cardiomyogenesis model associated with polysome profiling to investigate temporally regulated lncRNAs and their association with polysomes during human cardiac commitment.

2. Results

2.1 LncRNAs are expressed during cardiomyogenesis of hESCs

Models of in vitro differentiation of hESCs provide the ability to study developmental biology and address the key events that regulate early lineage commitment [26]. Our group used a developmentally staged protocol to induce cardiogenic commitment of hESCs [27,28] and perform polysome profiling, followed by RNA-seq, to investigate the differential association of RNAs with polysomes, as described in [29]. Here, we focused on analyzing lncRNAs expressed during cardiomyogenesis, mainly those differentially associated with polysomes. Considering an RPKM mean of at least 0.01 between biological triplicates (of each timepoint and distinct RNA fraction), 6693 lncRNAs were identified as being expressed during the five timepoints of cardiomyogenesis analyzed: days D0, D1, D4, D9 and D15 of differentiation, which represent pluripotency, embryoid body (EB) aggregation, cardiac mesoderm, cardiac progenitor and cardiomyocyte stages, respectively (Figure 1A). The lncRNAs identified during cardiomyogenesis represent 17.7% of the total genes expressed (Figure 1B), considering the filter mentioned above. The annotated categories included in this analysis were based on Ensembl gene biotypes: antisense, lincRNA, processed transcript, sense intronic, sense overlapping and TEC (to be experimentally confirmed), and the number of genes in each category is shown in Figure 1C. Principal component analysis (PCA) was performed for the lncRNAs according to the type of RNA fraction (ribosome-free or polysome-bound) (Figure 1D). Ribosome-free samples were dispersed and showed less similarity between the day of differentiation and biological replicates (Figure 1D, left panel). On the other hand, polysome-bound samples showed more distinct groups relative to the day of differentiation, indicating high similarity between polysomal lncRNAs in experimental replicates and more pronounced temporal expression (Figure 1D, right panel).



83
84 **Figure 1.** LncRNAs during human cardiomyogenesis. (A) Schematic representation of the cardiomyogenic differentiation protocol indicating the developmental stages and timing of sample collection. Polysome profiling was performed at each timepoint indicated, and ribosome-free and polysome-bound RNAs were sequenced [29] (n=3). (B) The number of genes identified in the five timepoints analyzed and in the ribosome-free and polysome-bound fractions (RPKM>0.01), classified according to protein-coding, long non-coding (lncRNA) and other RNA biotypes. (C) The number of genes identified in each lncRNA category (Ensembl). (D) Principal component analysis (PCA) of ribosome-free and polysome-bound samples at days D0, D1, D4, D9 and D15 (n=3).

92
93 2.2 Temporal expression patterns of lncRNAs during cardiomyogenesis

94
95 The polysome profiling approach allowed us to isolate and separately sequence polysome-bound and ribosome-free RNAs from cardiomyogenesis during the five timepoints. Comparisons between each differentiation timepoint and the preceding timepoint, considering an overall FDR of ≤ 0.05 and $-2 \geq \text{logFC} \geq 2$, identified differentially expressed (DE) lncRNAs in each RNA fraction. DE lncRNAs were plotted on a heatmap and showed a clear temporal expression pattern as either polysome-bound or ribosome-free RNAs (Figure 2). Hierarchical clustering grouped more highly expressed lncRNAs into distinct stages, and we classified them into six groups. For instance, some groups that showed high expression in cardiac committed cells (days D9 and D15) - group I; in the mesoderm stage (day D4) - group III; and in the pluripotency state (days D0 and D1) - group V. Not all groups were present in both the ribosome-free and polysome-bound fractions. Mesoderm stage group III only had a few genes in the ribosome-free fraction. Additionally, intermediate expression group IV, which had higher expression on days D1, D4 and D9, was only found in the polysome-bound fraction. Comparing the expression pattern of the ribosome-free and polysome-bound
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108 fractions, we observed more stated groups on polysome-bound lncRNAs and more progressive
109 patterns on ribosome-free lncRNAs (Figure 2). Interestingly, the temporal differential expression
110 observed in polysome-bound RNAs could suggest that lncRNAs differentially associate with
111 polysomes during cardiomyogenesis and that they play a role in this process.
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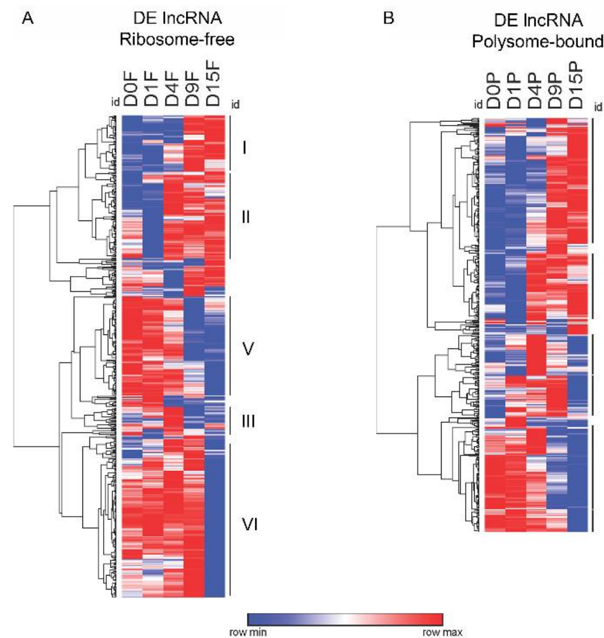

113
114 **Figure 2.** Differentially expressed lncRNAs during cardiomyogenic commitment. Heatmap of DE
115 lncRNAs during hESC cardiomyogenesis in the (A) ribosome-free and (B) polysome-bound fractions
116 (each differentiation timepoint was compared to the preceding timepoint, RPKM>0.01, FDR<0.05, -
117 2>logFC>2). I-VI groups established according to the expression pattern. The heatmap was made
118 using https://software.broadinstitute.org/morpheus/.
119

120 In addition, clustering analysis of lncRNAs showed particular expression patterns associated
121 with cardiomyogenic commitment (in the abovementioned time points: D0, D1, D4, D9, D15). Figure
122 3A shows the results of a PCA analysis of the genes of ribosome-free (right panel) and polysome-
123 bound (left panel) lncRNAs (with at least one CPM in three samples), in which 7 clusters were
124 identified (marked with an ellipse in the panels). The gene expression profiles of the 7 clusters for
125 both RNA fractions were determined (Supplementary Figure 1 and 2, polysomal and free,
126 respectively). Figure 3B shows the expression profiles of two example clusters of polysome-bound
127 lncRNAs. In the left panel, gene expression is high during the initial steps of cardiomyogenic
128 differentiation (D0, D1, D4), with a strong decay afterwards. The second cluster shows genes with
129 low expression during the initial steps of cardiomyogenic differentiation, with a peak at D4 and
130 stabilization during the final stages (right). Figure 3C shows two example clusters of lncRNA
131 expression in the ribosome-free fraction. These two clusters behave very similarly to those mentioned
132 above. To the left, a constant decrease of gene expression is observed from D4 onwards, and to the
133 right, a steady increase of gene expression is observed, with a peak at D4.
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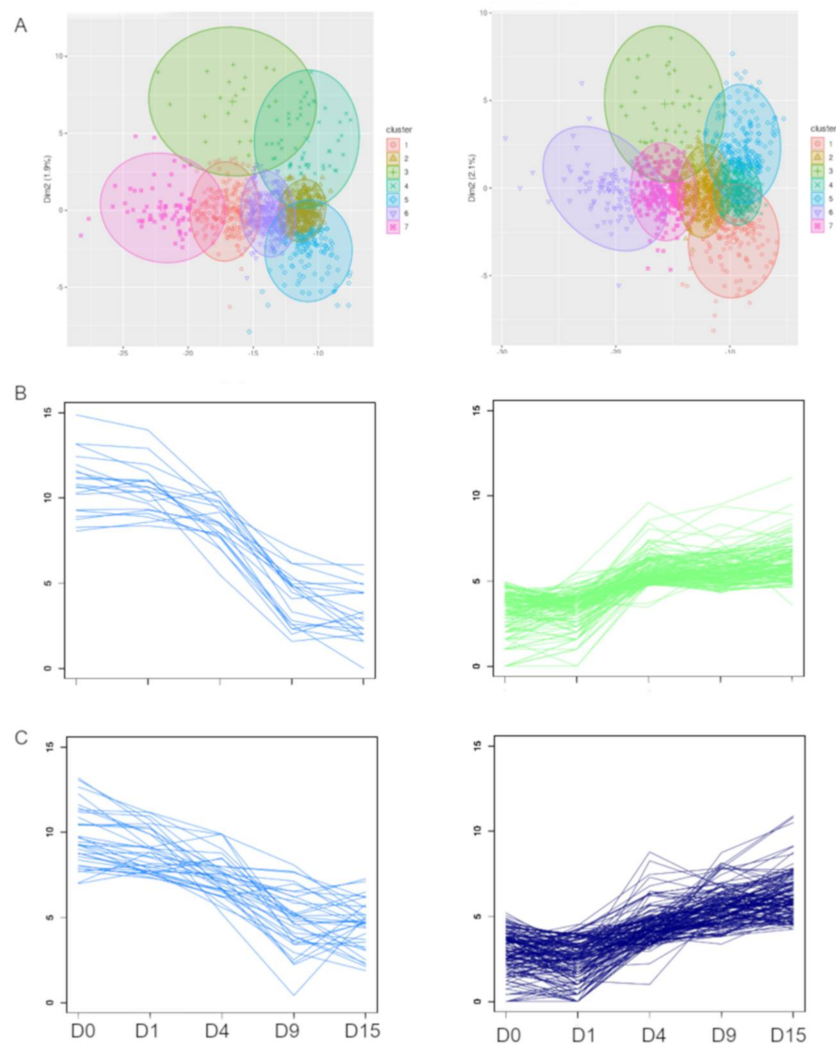

Figure 3. Gene expression pattern clusters of ribosome-free and polysome-bound lncRNAs during cardiomyogenesis. (A) PCA analysis of lncRNAs identified 7 distinct clusters (marked with an ellipse). Examples of clusters of polysome-bound (B) and ribosome-free (C) lncRNAs. In the left panel, gene expression is high in the initial steps of cardiomyogenic differentiation (D0, D1, D4) with a strong decay afterwards. In the right panel, genes expression is low expression in the initial steps and stabilize in the final stages.

Similarly behaving clusters of lncRNAs in the polysome-bound and ribosome-free fractions have lncRNAs in common, e.g., 38% in the case of the left ones and 14% in the case of the right ones (Figure 3B and 3C, left and right panels, respectively). Interestingly, several lncRNAs are specific to each fraction; the right panel clusters have 107 lncRNAs specific to the ribosome-free RNA fraction and 97 lncRNAs specific to the polysome-bound fraction (the Venn diagrams in Supplementary Figure 3A and 3B). Twenty out of 107 lncRNAs belong to clusters that show no change between time points in the polysomal fraction (Supplementary Figure 1, polysome-bound clusters 1, 2, 6). The rest of the lncRNAs are not even expressed in the polysomal fraction. Forty-five polysomal-specific lncRNAs belong to clusters that show no change in ribosome-free RNA (Supplementary Figure 2, ribosome-free clusters 2, 4 and 7). These results suggest a degree of post-transcriptional regulation of lncRNAs in cardiomyogenic differentiation. In addition, pathway analysis of the lncRNAs in each RNA fraction (polysome-bound and ribosome-free) was performed (Supplementary Table 1). Characteristic significant pathway terms (p-value<0.05) were observed in each fraction.

2.3 lncRNAs are recruited to polysomes during the cardiomyogenic commitment of hESCs

Considering the DE lncRNAs, we further investigated whether the lncRNA association with polysomes was only a consequence of transcriptional regulation, e.g., the more transcripts available, the more association with polysomes. We observed that some lncRNAs were only regulated in the polysome-bound fraction, only in the ribosome-free fraction or in both fractions, the latter representing coordinated regulation (Figure 4A). Interestingly, the majority of upregulated genes between days D0 vs D1 were only in the polysome-bound fraction, suggesting the recruitment of those transcripts to the polysome complex. Moreover, between days D9 vs D15, the majority of downregulated lncRNAs were only in the ribosome fraction, suggesting that some transcripts were held in the polysome-bound fraction when their transcription was decreased (Figure 4A). Essentially, all of the timepoints analyzed showed that more lncRNAs were regulated in only either the polysome-bound or the ribosome-free fraction than in both fractions (2.5 to 40% of lncRNAs). This result could represent a specialized regulation of lncRNA association with polysomes, independent of the transcript abundance.

Once we observed there was differential lncRNA association with polysomes, we asked whether non-DE lncRNAs were also differentially recruited to and dissociated from polysomes during cardiomyogenesis. We calculated the ratio between the polysome-bound and ribosome-free RPKM values to assess the prevalence of lncRNA in each fraction. The results showed that some lncRNAs were affected by polysome recruitment (FDR $\leq$ 0.05, logFC $\geq$ 2) or dissociation (FDR $\leq$ 0.05, -2 $\geq$ logFC) when comparing each differentiation timepoint and the preceding timepoint during cardiomyocyte differentiation (Figure 4B).

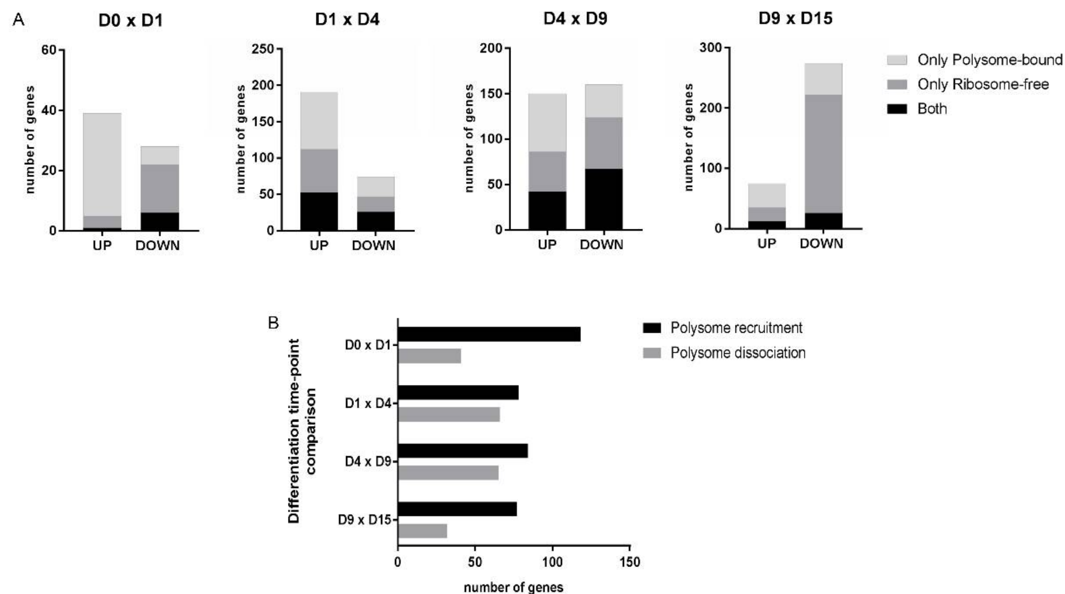

Figure 4. LncRNAs are associated with polysomes during cardiomyogenesis. (A) LncRNAs that are differentially expressed ($RPKM > 0.01$, $FDR < 0.05$, $-2 > logFC > 2$) only in the polysome-bound, ribosome-free or both fractions (coordinated). (B) The number of non-DE lncRNAs recruited to and dissociated from polysomes (ratio poly/ribo-free, $FDR < 0.05$, $-2 > logFC > 2$).

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3. Discussion

Cardiomyogenesis of hESCs provides an exceptional model to investigate the mechanisms of cell fate determination [26]. In this report, we analyzed five distinct timepoints during the differentiation of hESCs into cardiomyocyte to assess the expression of lncRNAs involved in the temporal cell fate transition. Our results identified a large amount of lncRNAs in both ribosome-free and polysome-bound fractions during cardiogenesis (Figure 1). LincRNA and antisense lncRNA were the most representative categories identified in our data, corroborating previous findings that antisense lncRNA may represent one of the largest lncRNA biotypes in mouse and human genomes [30]. The functions of antisense lncRNA are not yet well understood, but because they share sequence similarities with their sense transcripts, it is believed that they could regulate their sense transcripts at transcriptional or post-transcriptional levels [10,30], for instance, processing the sense strand by differential splicing or editing [31].

Transcriptome studies have revealed thousands of differentially expressed lncRNAs during cardiomyocyte differentiation [19–23], and some evidences have demonstrated the crucial roles that lncRNAs play in heart development [32]. The murine lncRNAs Braveheart and Fendrr are classical examples of lncRNAs that function in heart development [33,34]. These lncRNAs interact with the repressor complex Polycomb 2 and regulate gene expression during development. In humans, the antisense transcript of the Braveheart ortholog has been shown to be essential in cardiac determination, acting by enhancing activity [35]. More recently, murine linc1405 was described in cardiac differentiating cells [36]. Murine linc1405 binds to the transcription factor Eomes and the histone modifiers WDR5 and GCN5 to form a complex that controls Mesp1 expression. These examples illustrate that lncRNAs can act as regulators of gene transcription in cardiac differentiation. However, evidence showing their involvement in other modes of gene regulation, e.g., controlling mRNA translation, is still limited.

Here, we used polysome profiling and RNA-seq of ribosome-free and polysome-bound RNAs to investigate lncRNAs that were differentially associated with polysomes. Polysome profiling analysis is a robust method that has been used to provide valuable information regarding the association of ribosomes with RNAs [14,37,38]. Principal component analysis showed that polysome-bound lncRNAs were able to better reflect the biology of the samples than ribosome-free lncRNAs regarding the day of differentiation (Figure 1D). Additionally, comparisons between each differentiation timepoint and the preceding timepoint showed dramatic differential expression of lncRNAs during cardiogenesis, illustrating the molecular complexity of cardiac differentiation (Figure 2). Interestingly, we observed a very specific temporal expression of polysome-bound RNAs, suggesting not only that lncRNAs could differentially associate with polysomes during cardiomyogenesis but also that they could play a crucial role in this process. It has already been shown that lncRNAs can be found in polysomal complexes [13–16]; however, their function remains uncharacterized. In addition to their coding potential (e.g., small peptides) [17,39], the association of lncRNAs with polysomes could represent an additional cytoplasmic role in mRNA metabolism, for instance, in translational regulation by acting at the ribosome or mRNA level [1,7,11].

Moreover, we found that similar behaving clusters of lncRNAs in polysome-bound and ribosome-free fractions had few lncRNAs in common (Supplementary Figure 3), and all of the timepoints analyzed showed that more lncRNAs were only regulated in either the polysome-bound or only ribosome-free fraction (Figure 4A). Taken together, these results suggest that the association of lncRNAs with polysomes is independent of the transcript abundance and that specific lncRNAs are able to interact with the polysomal complex. Polysomal lncRNAs could act at the post-transcriptional level of gene expression regulation in different ways. Characterization of the post-transcriptional functions of lncRNAs is still being performed [40]. In human carcinoma HeLa cells, lincRNA-p21 was shown to repress the translation of mRNAs encoding β-catenin and JunB by partial base-pairing and recruitment of translation repressor proteins [41]. During cell differentiation, the lncRNA TINCR interacts with the STAU-1 protein to form a complex that mediates the stabilization of differentiation mRNAs [42]. Additionally, linc-31 is required to stabilize the translational activator YB-1 and promote ROCK1 protein synthesis, which controls the proliferation to differentiation switch

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in myoblasts [43]. Recently, the lncRNA Airn, which is known to induce the imprinting of genes on its locus [44], was shown to also act in in the cardiomyocyte cytoplasm. A spliced Airn isoform was found bound to the Igf2bp2 transcript and to be involved in the translation control of Igf2bp2 and a number of other genes [45].

Technological advances, including RNA-seq and computational analyses, have allowed improved characterization of non-coding RNAs [32]. These dynamic molecules seem to contribute to the complexity of organisms because non-coding sequences are far more numerous than protein-coding sequences in humans compared to the worm or fruit fly [32,46]. In this report, we characterized the dynamic expression patterns of lncRNAs during cardiomyogenesis and identified a stage-specific pattern expression. LncRNAs expressed in differentiating cells are likely to be involved in cell specialization pathways, while cardiomyocyte lncRNAs might play roles in maturation and cell-fate maintenance. Because RNAs can be rapidly transcribed and degraded, they represent a type of molecule that is perfectly suitable for regulatory mechanisms. In addition, the sequence and structure complexity of lncRNAs allow a plethora of ligand binding that can broaden their functions [31]. Despite some advances, most lncRNAs remain uncharacterized, and it will be important to investigate their mechanisms of action, targets and gene networks in the future.

Taken together, our findings support a role for lncRNAs in the temporal cell fate transition of pluripotent stem cells into cardiac precursor cells and cardiomyocytes.

4. Materials and Methods

4.1 Cell culture and cardiomyocyte differentiation

The NKX2-5eGFP/w HES3 cell lineage was donated by Monash University (Victoria, Australia) [47] and cultured as previously described [29]. hESCs were submitted to a cardiac differentiation protocol adapted from [27,28] and described in detail in [29]. Briefly, cells were mildly dissociated to form small clusters; cultured in 6-well Ultralow Attachment Corning plates using StemPro-34 (Invitrogen) supplemented with 100 μg/ml penicillin-streptomycin, 2 mM L-glutamine, 150 μg/mL transferrin, 50 μg/mL ascorbic acid and 0.45 mM monothioglycerol (MTG); and kept in a humid incubator at 37°C in 5% CO2 and 5% O2. For embryoid body (EB) aggregation (D0-D1), the basal medium was supplemented with 1 ng/mL BMP4. On D1, the medium was replaced with medium supplemented with 10 ng/mL BMP4, 6 ng/mL Activin A and 5 ng/mL bFGF; on D4, with medium supplemented with 10 μg/mL VEGF and 10 μM XAV 939. Starting on D8, the medium was replaced every two or three days with medium supplemented with 10 μg/mL VEGF and 1 ng/mL BMP4. Three independent differentiation assays were used as experimental replicates.

4.2 Samples and sequencing

As previously described [29], monolayer hESCs on D0 or differentiating EBs (on D1, D4, D9 and D15) were treated with 0.1 mg/mL cycloheximide, and cell lysates were loaded onto 10% to 50% sucrose gradients and centrifuged at 150000 x g for 160 minutes at 4°C. Different sucrose gradient fractions were separated using the ISCO gradient fractionation system (ISCO Model 160 Gradient Former Foxy Jr. Fraction Collector). Ribosome-free and polysome-bound fractions were isolated and used to prepare cDNA libraries. RNA-seq was carried out on an Illumina HiSeq platform.

4.3 Analysis of sequencing data

Reads were mapped against the reference genome GRCh38 with Rsubreads, and features were counted using the function featureCounts as previously described [29]. Biotypes were annotated using the Ensembl database (Ensembl Genes 78). Only lncRNAs and antisense transcripts with at least one CPM in at least three samples were considered for further analyses. K-means clustering analysis was performed on the matrix of CPMs, with k equal to 7. Expression profiles were visualized, and genes belonging to the clusters were further analyzed separately. Pathway analysis of the lncRNAs in each RNA fraction (polysome-bound and ribosome-free) was performed using toppGene Suite [48]. The polysome/ribosome-free ratio was determined by dividing the RPKM values derived

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288 from the polysomal fraction by those derived from the ribosome-free fraction (three biological
289 replicates were averaged).

290 **Supplementary Materials:** Figure S1: Gene expression profiles of the 7 clusters of polysome-bound lncRNAs.
291 Figure S2: Gene expression profiles of the 7 clusters of ribosome-free lncRNAs. Figure S3: Venn diagrams
292 showing specific and common lncRNAs from (A) Figure 3, left panels, and (B) Figure 3, right panels. Table S1:
293 Pathway analysis of the lncRNAs in each RNA fraction. The ribosome-free fraction is on top with the name of
294 the enriched pathway term in the first column, the source from which the information was taken in the second
295 column, FDR in the third column, the number of genes from the input found in the database in the fourth column,
296 and the genes name in the fifth column. The polysome-bound fraction is shown on the bottom.

297 **Author Contributions:** ITP performed the experiments. LS and GC performed the bioinformatic analyses. ITP,
298 LS and BD wrote the manuscript. BD conceived and coordinated the work.

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305 **Conflicts of Interest:** The authors declare no conflict of interest.

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