Despite recent developments in the treatment of myocardial ischemia, ischemic heart disease still remains the leading cause of death. It is caused by atherosclerosis in the coronary arteries, and an acute myocardial infarction (AMI) is its most severe complication. After AMI, without fast and successful revascularization, the compromised myocardium is replaced by scar tissue, impairing the ventricular function. Also, the injured myocardium predisposes the heart for arrhythmias, such as ventricular fibrillation, and can thus lead to sudden cardiac death. The pathological remodeling of the affected myocardium is thought to result from maladaptive transcriptional reprogramming. Deeper understanding of the gene regulatory processes that control this process is needed to develop novel treatment strategies.

Background—Microarrays and RNA sequencing are widely used to profile transcriptome remodeling during myocardial ischemia. However, the steady-state RNA analysis lacks in sensitivity to detect all noncoding RNA species and does not provide separation between transcriptional and post-transcriptional regulations. Here, we provide the first comprehensive analysis of nascent RNA profiles of mRNAs, primary micro-RNAs, long noncoding RNAs, and enhancer RNAs in a large animal model of acute infarction.

Methods and Results—Acute infarction was induced by cardiac catheterization of domestic swine. Nuclei isolated from healthy, border zone, and ischemic regions of the affected heart were subjected to global run-on sequencing. Global run-on sequencing analysis indicated that half of affected genes are regulated at the level of transcriptional pausing. A gradient of induction of inflammatory mediators and repression of peroxisome proliferator-activated receptor signaling and oxidative phosphorylation was detected when moving from healthy toward infarcted area. In addition, we interrogated the transcriptional regulation of primary micro-RNAs and provide evidence that several arrhythmia-related target genes exhibit repression at post-transcriptional level. We identified 450 long noncoding RNAs differently regulated by ischemia, including novel conserved long noncoding RNAs expressed in antisense orientation to myocardial transcription factors GATA-binding protein 4, GATA-binding protein 6, and Krüppel-like factor 6. Finally, characterization of enhancers exhibiting differential expression of enhancer RNAs pointed a central role for Krüppel-like factor, MEF2C, ETS, NFY, ATF, E2F2, and NRF1 transcription factors in determining transcriptional responses to ischemia.

Conclusions—Global run-on sequencing allowed us to follow the gradient of gene expression occurring in the ischemic heart and identify novel noncoding RNAs regulated by oxygen deprivation. These findings highlight potential new targets for diagnosis and treatment of myocardial ischemia. (Circ Cardiovasc Genet. 2017;10:e001702. DOI: 10.1161/CIRCGENETICS.117.001702.)

Key Words: gene expression ■ microRNAs ■ myocardial infarction ■ myocardial ischemia ■ RNA, long noncoding

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Recently, hundreds of heart-specific lncRNAs with potential regulatory and functional roles were identified in response to AMI in mouse.⁶ Information on immediate transcriptional changes after the onset of myocardial ischemia is needed to fully understand the gene regulatory processes taking place in response ischemic insult. However, to the authors’ knowledge, such analysis has never been performed. In this study, we examined acute transcriptional changes in response to myocardial ischemia using a global run-on sequencing (GRO-Seq) after AMI in a clinically relevant large animal AMI model. In contrast to traditional RNA sequencing, GRO-seq provides us data that distinguish primary transcription from post-transcriptional processing while offering all-in-one form of sequencing, in which the relative unstable ncRNAs can be measured simultaneously to the precursor mRNA expression. To this end, we studied the gradual changes in nascent RNA expression when moving from healthy areas to the border of the ischemic zone and ultimately to the completely ischemic zones, revealing gradual changes in the transcription of different precursor mRNAs related to inflammation, peroxisome proliferator-activated receptor (PPAR) signaling, and oxidative phosphorylation. In addition, we identified several differentially regulated ncRNA species, including primary (pri)-miRNAs, IncRNAs, and recently discovered enhancer RNAs (eRNAs). Many of these ncRNAs are orthologous to human genes and provide new insights into the pathological responses to AMI. Further characterization of these ncRNAs could reveal new targets for diagnosis and therapeutic interventions.

Methods

Animal Experiments
All animal experiments in this study were approved by National Experimental Animal Board of Finland. Female farm pigs underwent cardiac catheterization under general anesthesia. Microcatheter was used to place an embolization coil into distal left anterior descending artery leaving the first and the second diagonal branches open (Figure 1A and 1C). The occlusion was confirmed by ST-elevation and angiography. Detailed description of the animal experiments, immunohistochemistry, and protein analysis can be found in the Data Supplement.

Cell Culture
Extraction of neonatal ventricular cardiomyocytes was performed as previously described.⁷ Detailed description of the cell culture conditions can be found in the Data Supplement.

RNA Isolation and Quantitative Polymerase Chain Reaction
Tissue samples were homogenized using Precellys 24 homogenizer, and RNA was extracted using Trizol (Thermo Fisher Scientific). RNA from mouse cardiomyocytes was isolated with E.Z.N.A Total RNA Kit (Omega Bio-Tek, GA). RNA extracts were treated with DNase I and reverse transcribed using RevertAID or Taqman MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed on StepOnePlus Real-Time PCR System using Power SYBR Green PCR Master Mix or TaqMan miRNA Assay (Thermo Fisher Scientific). Detailed description of qPCR analysis can be found in the Data Supplement. The primer and miRNA assay information is listed in Table 1A and 1B in the Data Supplement, respectively.

Figure 1. Occlusion of the left anterior descending artery (LAD) resulted in formation of hypokinetic area in the anterior wall of the left ventricle characteristic of ischemia. A, Angiogram of the left coronary artery (LCA) at baseline, and LAD with 2 first diagonal branches is shown in the zoom box. The LAD is patent. B, Left ventricular cine angiography (LV-CINE) at baseline. The white lines show the range of contraction. The anterior and posterior walls both contract well. C, Angiogram of the LCA 24 h after coil placement. LAD is occluded proximally to the coil. Two first diagonal branches are left patent. Placed coil is shown in the zoom box. D, LV-CINE 24 hours after the coil placement. Clear akinesia of the anterior and apical wall is visible. Also, the coil occluding the LAD can be seen above the hypokinetic area.
Global Run-On Sequencing
The heart tissue was minced and collagenase treated to obtain single cell suspension. This was followed by nuclei extraction, nuclear run-on reaction, and library preparation. Detailed description of the GRO-Seq protocol can be found in the Data Supplement.

Data Analysis
Data analysis was performed using HOMER 4.3, and detailed instructions for analysis are found at http://homer.salk.edu/homer and in the Data Supplement.

Data Access
Experiments performed and public data used in this study are available in gene expression omnibus under the accession number GSE81155 and GSE51169, respectively.

Results
Differentially Expressed Genes Are Related to Inflammation, Mitochondrial Dysfunction, and PPAR Signaling
To study acute infarction in pigs, we occluded the left anterior descending artery using an embolization coil which resulted in occlusion 24 hours after the procedure in all animals studied (Figure 1A and 1C). Also in left ventricular cine angiography, a clear hypokinetic area was observed in the anterior wall of the left ventricle at day 1 when compared with baseline (Figure 1B and 1D). The newly infarcted tissue was clearly detectable in hematoxylin eosin stain (Figure I in the Data Supplement), and incipient fibrous tissue formation was seen in the Masson’s trichrome staining (Figure I in the Data Supplement). The normal vascular structures were not present in the infarcted areas (Figure 1 in the Data Supplement), and there was also marked apoptosis in the ischemic areas, as detected by cleaved caspase 3 immunostaining (Figure I in the Data Supplement).

To study the acute changes in gene expression, we performed GRO-Seq from nuclei isolated from different parts of the affected heart 1 day after ischemia operation. GRO-Seq allows the detection of nascent RNAs, thus enabling more reliable estimation of changes occurring at transcriptional level compared with RNA sequencing or microarrays that are also affected by post-transcriptional regulation. In addition, GRO-Seq enables identification of all actively transcribed RNAs, including lowly expressed ncRNAs such as eRNAs. Total of 913 annotated precursor mRNAs were differentially regulated (reads per kb per million reads >1, fold change >2, and false discovery rate <0.1) within our data set (Figure IIA in the Data Supplement; Table IC through IE in the Data Supplement), and a clear gradient of gene induction and repression was seen when moving from healthy to more hypoxic border zone and eventually to ischemic area (Figure 2A). Accordingly, the changes in gene expression between the border zone and the ischemic area compared

**Figure 2.** Gene expression changes are characterized by a gradient of induction of inflammatory mediators and repression of peroxisome proliferator-activated receptor (PPAR) signaling and oxidative phosphorylation when moving from healthy toward infarcted area of the pig myocardium. A, Hierarchical clustering and heat map of the normalized gene expression values (RPKM) for the differentially regulated genes (RPKM >1, fold change >2, and FDR <0.1). The 3 replicates for each condition are shown. B, Scatter plot of the fold changes in gene expression comparing the border zone and ischemic zones to healthy areas. The genes showing opposite regulation between the 2 areas are highlighted in red and green. The Pearson correlation value (r) is also shown. C, Gene ontology analysis of the groups of genes showing induction or repression of gene expression when moving from healthy toward ischemic tissue. D, Representative Western blot images of transforming growth factor β-1 (TGFβ1) and adenosine A1 from the pig heart tissue lysates. Equal loading was confirmed using antibodies against GAPDH. The increase in ischemia when moving from healthy (left) to border zone (middle) and to infarcted tissue (right) is indicated by a triangle. ADORA1 indicates adenosine A1 receptor; NOD, nucleotide-binding oligomerization; and TCA, tricarboxylic acid.
with healthy area showed a strong correlation (Figure 2B). Furthermore, the 3 replicates used in the analysis clustered well together based on the tissue type (Figure II B in the Data Supplement). The gene ontology analysis indicated that induced genes were largely related to inflammation as exemplified by the induction of transforming growth factor β (TGF-β) and repression in the ischemic zone. Same was observed for SLCA25A51 exhibited induced gene expression in the border zone but repression in the ischemic zone. On contrary, 2 solute carrier family members SLC31A2 and SLC25A51 exhibited induced gene expression in the border zone but repression in the ischemic zone. Same was observed for A1 adenosine receptor (ADORA1; LOC606743) where higher expression in the border zone might increase myocardial resistance to ischemia, suggesting that downregulation of these transcription factors could present a protective response, which is lost on ischemia. On contrary, 2 solute carrier family members SLC31A2 and SLC25A51 exhibited induced gene expression in the border zone but repression in the ischemic zone. Same was observed for A1 adenosine receptor (ADORA1; LOC606743) where higher expression in the border zone might increase myocardial resistance to ischemia, suggesting that downregulation of these transcription factors could present a protective response, which is lost on ischemia.

Among the regulated genes, we also identified several factors with opposite regulation within the border and ischemic zones, namely repression in the former and induction in the latter. These included bone morphogenic protein 4, transcription elongation factor A (SII)-like 4, and GATA-binding protein 2 (GATA2). Of these, bone morphogenic protein 4 has been previously shown to mediate myocardial ischemic injury, suggesting that downregulation of these transcription factors could present a protective response, which is lost on ischemia. On contrary, 2 solute carrier family members SLC31A2 and SLC25A51 exhibited induced gene expression in the border zone but repression in the ischemic zone. Same was observed for A1 adenosine receptor (ADORA1; LOC606743) where higher expression in the border zone might increase myocardial resistance to ischemia, suggesting that downregulation of these transcription factors could present a protective response, which is lost on ischemia.

Half of the Genes Are Regulated at the Level of Transcriptional Pausing

Recent studies have shown that the pausing of polymerase II (Pol II) in promoter-proximal regions and its release into productive elongation are key steps regulating transcription in response to cardiac hypertrophy. By measuring nascent transcription, we were also able to interrogate the role of this phenomenon in ischemia response. In contrast to Chromatin immunoprecipitation (ChIP) followed by high-throughput DNA, which measures the density of Pol II that is bound to chromatin, GRO-Seq has the advantage of measuring Pol II that is elongation competent. To this end, we calculated the pause ratio as a measure of relative ratio of Pol II density in the promoter-proximal region (0–200 bp) and the gene body (200 bp to end of gene) (Figure 3A). We found that ≈50% of the induced genes and ≈35% of the repressed genes of the border zone displayed >2-fold decrease or increase in pause ratio, respectively, as exemplified by chemokine (C-C motif) ligand 2 gene (Figure 3A and 3B). This was further increased to ≈60% of the induced gene and ≈55% of the repressed genes within the ischemic area. A similar degree of decrease was observed in the pause ratio of induced genes when moving from healthy to border or to ischemic zones (Figure 3A and 3C). However, a proportionally higher increase in pause ratio was observed in the ischemic zone compared with border zone (Figure 3A and 3C).

Interestingly, gene ontology analysis revealed that majority of the repressed genes that undergo incremental increase in pausing belong to the mitochondrial membrane complexes, such as NADH:ubiquinone oxidoreductase subunits (NDUF), ATP synthases 5, and cytochrome c oxidase, and are enriched for pathways implicated in muscle contraction (Table I in the Data Supplement). In contrast, the induced genes undergoing a decrease in pausing belong to different stages of the unfolded protein response or inflammatory cascade and accordingly are enriched for pathways related to necrosis and cellular movement. Inflammatory signaling being one of the major pathways induced by myocardial ischemia, we also took advantage of public Pol II ChIP-Seq data from AC16 human cardiomyocytes subjected to tumor necrosis factor-α for 24 hours and studied the pause ratios of genes induced in this data set and ours. The results showed that ≈60% of inflammatory genes display >2-fold decrease in pause ratio in human cardiomyocytes on inflammatory

![Figure 3](https://example.com/figure3.png)
stimuli, providing evidence of the broader applicability of the results also in humans (Figure III in the Data Supplement). Altogether, our results suggest that promoter-paused Pol II plays a role in regulating a specific subset of genes during ischemic response, whereas the remainder of gene exhibits more global changes in GRO-Seq signal within the promoter-proximal region and gene body indicative of regulation at the level of initiation and rate of transcription (Figure 3D).

Identification of pri-miRNA Transcripts

We next studied the expression of ncRNAs in our model. For the first time, we were able to identify the pri-miRNA coordinates in heart tissue and interrogate the changes in their expression during myocardial ischemia. Our analysis led to the identification of 126 expressed pri-miRNAs (RPKM >0.5) with a median size of 20 kb and corresponding to 169 individual miRNAs (Table II in the Data Supplement). Of these, 55 pri-miRNAs were found differentially regulated (RPKM >0.5, fold change >2, and FDR <0.1) between healthy, border, and ischemic areas, as exemplified MIR21 (Figure 4A and 4B; Table II in the Data Supplement). Despite our inability to discriminate between the different cell types of the heart, our observation that the most highly expressed downregulated miRNAs include the highly cardiomyocyte-specific miR1, miR133, and miR208 suggests that cardiomyocytes likely represent the major cell type in our tissue analysis. Over half of human homologs of the identified miRNAs have been shown to be regulated in miRNA profiling studies for atrial fibrillation (Figure 4A marked with *).

The expression of pri-miRNAs exhibited good correlation with mature miRNAs differentially regulated in human myocardial infarction (Figure 4C; Figure III in the Data Supplement; Table II in the Data Supplement). In addition, we verified the correlation between 12 of the pri-miRNAs and

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**Figure 4.** Identification of differentially regulated primary micro-RNA (miRNA) transcripts and their predicted targets. A, Hierarchical clustering and heat map of the normalized gene expression values (RPKM) for differentially regulated primary (pri)-miRNAs (RPKM >0.5, fold change >2, and FDR <0.1). The 3 replicates for each condition are shown. The miRNAs differentially regulated in atrial fibrillation are marked by an asterisk. The upregulated and downregulated pri-miRNAs are listed in the order of decreasing maximum expression (RPKM). B, UCSC Genome browser image depicting normalized global run-on sequencing (GRO-Seq) tag counts for primary miR21 transcript. C, Comparison of fold changes in pri-miRNA expression of the ischemic pig myocardium and mature miRNA expression in human myocardial infarction. The Pearson correlation coefficients are shown. D, Comparison of fold changes in pri-miRNA and mature miRNA expression. The results are represented as mean fold changes of 3 samples.
mature miRNAs by qPCR (Figure 4D; Table IJ in the Data Supplement). Strikingly, the downregulated pri-miRNAs, miR-499, miR-133a, miR-208b, miR-103, and let-7c, exhibited no clear repression at the level mature miRNA level in 24-hour old infarcted pig tissue, whereas this was clearly seen in <7 days old infarcted human tissue (Figure 4C and 4D; Figure IV in the Data Supplement).

We used ComiR-tool18 to predict common mRNA targets among the significantly regulated genes for the 18 upregulated miRNAs and 37 downregulated miRNAs by incorporating pri-miRNA expression level in the prediction of target occupancy (Figure 5A; Table IK in the Data Supplement). As expected, the top-scored mRNAs were targeted by several different miRNAs, suggesting that the fine balance between the miRNAs is likely to determine the direction of regulation (Table IL in the Data Supplement). This is exemplified by vinculin (VCL) and sprouty homologue 1, which have 29 and 13 different miRNA-binding sites and exhibit downregulation at the level of pri-miRNA, respectively (Figure 5A).

The repression of these genes was further confirmed on protein level (Figure 5B). The Combinatorial miRNA targeting score did not correlate with either repression or induction of target mRNA expression, confirming that majority of miRNA effects are likely to occur on post-transcriptional level. To study the effect on mature mRNA levels, we selected 10 genes for further analysis of mature mRNA levels by qPCR. These include 6 genes previously linked to arrhythmias, such as VCL, BTG family member 2, sofilin 2, superoxide dismutase 2, gap junction protein α1, and prostaglandin-endo-peroxidase synthase 2. Comparison of the fold changes in expression compared with GRO-Seq revealed that majority of the genes (8 of 10) exhibited further repression at the level of mRNA expression (Figure 5C).

Identification of Novel IncRNAs Regulated by Ischemia

Extensive studies of long IncRNAs have demonstrated that they can regulate gene expression during development and disease, acting both as transcriptional repressors and activators. Several IncRNAs have also been found regulated after myocardial infarction in patients,19 and a recent study in mice revealed novel heart-specific IncRNAs with functional characteristics relevant to maladaptive remodeling, cardiac function, and cardiac regeneration.6 We therefore sought to identify differentially regulated IncRNAs in a large animal model, with a more human-like cardiac physiology. Our analysis focused on intergenic IncRNAs because their further analysis is not complicated by the sense strand transcription; however, we acknowledge that many IncRNAs were also found within introns of genes (data not shown and Figure 6C). We identified 3858 high-confidence IncRNAs of which 3503 could be converted to human genomic coordinates (Table IM in the Data Supplement). Of these, 55% (1912) overlap human-coding genes (NM_RefSeq) and are likely genes missing from the current SusScr3 genome annotation, whereas 18% (292 NR_RefSeq, 322 InciPedia=614) are orthologous to human ncRNAs (Figure VA in the Data Supplement). Seventy-one percent (693 of 977) of the remainder overlapped to de novo transcripts detected from public GRO-Seq performed in human cardiomyocyte cell line AC16 and thus represent novel IncRNAs found also in man (data not shown).

We next sought to identify IncRNAs differentially regulated by ischemia. We first studied the expression of known IncRNAs previously shown to be regulated in patients with myocardial infarction (ie, myocardial infarction–associated transcript, metastasis-associated lung adenocarcinoma transcript 1, hypoxia-inducible factor 1A antisense RNA 2, and...
cyclin-dependent kinase inhibitor 2B antisense RNA 1; Figure 6A and 6B). The 3 latter were already induced in the border zone, whereas all 4 were found upregulated in the ischemic tissues, thus supporting the validity of our animal model. Of all the potential lncRNAs, 28% (449 of 1591; Figure VA in the Data Supplement) were differentially regulated (RPKM >1, fold change >2, and FDR <0.1), representing significantly higher portion of genes than among the RefSeq genes (hypergeometric test P value, 1.9e-6; Table IN in the Data Supplement). Similarly to the coding genes, the gene expression changes within the border and ischemic areas showed a strong correlation (Figure 6A). We identified several novel lncRNAs expressed in an antisense orientation to myocardial transcription factors, including GATA4, GATA6, and Krüppel-like family of transcription factor 6 (KLF6) (Figure 6A and 6C). In all cases, the antisense expression followed that of the coding gene, that is, upregulation of GATA4/6 and repression of KLF6 expression, suggesting a common mechanism of transcriptional regulation. All 3 lncRNAs were also found expressed in the human cardiomyocyte cell line AC16, suggesting that they are conserved between species. We also studied the expression of these lncRNAs in mouse neonatal cardiomyocytes subjected to 24-hour hypoxia. We found that KLF6 and GATA4 lncRNAs were expressed in primary mouse cardiomyocytes and responsive to hypoxia treatment (Figure VB in the Data Supplement). Altogether, the lncRNAs were found close to annotated pig genes implicated in integrin, phosphoinositide 3-kinase and mechanistic target of rapamycin signaling, and cardiomyocyte differentiation through bone morphogenic protein receptors (Figure 6D). Computational mapping of these regions to human genome-positioned lncRNAs close to genes implicated in cardiac hypertrophy as well as calcium, RhoA, and phospholipase C signaling (Figure 6E). This is in accordance with the link between ischemic insult and impaired...
calcium handling, which is a distinct and well-validated pathological process behind heart failure and is also recognized as an arrhythmogenic mechanism associated with heart failure.21

**eRNA Expression Marks Gene Regulatory Regions**

Recent studies have led to the unexpected finding that many enhancers direct the expression of RNA transcripts (eRNAs), in a manner that is correlated with the expression of nearby genes.22 There is emerging evidence suggesting that at least some eRNAs contribute to enhancer function by mediating enhancer–promoter contacts and by releasing polymerase from the promoter-proximal pausing.23 Notably, GRO-seq is currently one of the best methods for eRNA detection within gene regulatory regions and can be thus used as a surrogate for ChIP-seq to assess the sites of transcription factor binding and action. We identified ≥3700 bidirectional eRNA loci (ie, a total of ≥7400 eRNA transcripts; Table IO in the Data Supplement). Of these, ≥1700 were differentially expressed (RPKM >1, fold change ≥2; P<0.05), and their expression correlated with the closest gene expression (Figure 7A and 7B). Because enhancers contain the majority of binding sites for regulatory transcription factors, we performed de novo motif analysis around transcriptional start sites of differentially regulated eRNAs to identify possible regulatory transcription factor–binding sites. The results identify KLFs (similar to SP1) as the major regulators of enhancer activity during ischemia (Figure 7C). Interestingly, 3 of the KLF family members were also differentially regulated in our data set, KLF6 and KLF10 being induced and KLF12 repressed, when moving from healthy myocardium toward border zone and ischemic areas (Table IC and ID in the Data Supplement). Other transcription factor candidates include MEF2C, ETS, NFY, ATF, E2F2, and NRF1, which several have been shown to play a role in transcriptional responses to ischemia or mitochondrial biogenesis (Figure 7C).24–26 Interestingly, tracking of the eRNA signal centered around the respective transcription factor motif suggested that in most cases, the eRNA expression is induced by the TF (Figure 7D). However, MEF2C motif was mostly associated with repression in eRNA expression and thus decreased enhancer activity.

**Discussion**

In this study, we show that the expression levels of hundreds of nascent RNAs are regulated after myocardial infarction in a large animal model, the hallmarks being induction of inflammatory markers and repression of genes implicated in oxidative phosphorylation, cardiac muscle contraction, and PPAR signaling. This is in accordance with literature on the molecular mechanisms of myocardial ischemia in man.27 Our analysis clearly captures a gradient of gene expression when moving from healthy toward the ischemic zone. A similar incremental change is also seen in the pause ratios of affected genes, suggesting that half of them are controlled by the transition between paused and elongating forms of Pol II, whereas the remainders of the genes are mostly regulated at the level of initiation or rate of transcription seen as global change in GRO-Seq signal. The release of the polymerase into productive elongation is mediated by positive transcription elongation factor b, which has recently been shown to be recruited to hypoxia-induced genes by hypoxia-inducible factor 1α and subsequent binding of mediator-associated kinase CDK8.28 On the other hand, enhanced formation of inactive complex of positive transcription elongation factor b with its inhibitor Hexamethylene Bisacetamide Inducible 1 has been shown to promote transcriptional repression under acute hypoxia.29 Further studies are needed to determine whether the same transacting factors interacting with positive transcription elongation factor b could be responsible for coordinating the changes in transcriptional pausing in the ischemic myocardium. Interestingly, recent studies have reported that BRD4, a bromodomain protein that recognizes acetylated histones and recruits positive transcription elongation factor b to promoters, also mediates transcriptional pause release in heart failure and AMI, and pharmacological inhibition of BRD4 suppresses pathological cardiac remodeling by inhibition of inflammatory signaling.30,31 This further highlights the important role of the regulation of pause release in the process leading to myocardial infarction damage.

We also identified a handful of genes exhibiting a reverse regulation within the border zone compared with the ischemic zone. Among these, we find the A1 adenosine receptor that regulates myocardial oxygen consumption and coronary blood flow. The repression of receptor expression might inhibit adenosine signaling, thus leading to arrhythmias. To this end, adenosine has been previously linked to supraventricular tachycardia and ventricular tachycardia (VT). It is known that VTs based on re-entry mechanism do not respond to adenosine; however, focal VT often does, and adenosine response is used as a diagnostic tool to differentiate between re-entry and focal VTs.32 VTs are more common in patients with myocardial ischemia and manifest during acute ischemia and later as a results of re-entry because of myocardial scar.33 Increased expression of adenosine receptor A1 in the peri-infarct border zone might play a role in the adenosine responsiveness of certain types of VT because the peri-infarct region has been identified as an important arrhythmogenic source,34 and the increased size of the peri-infarct region is associated with higher mortality in patients with myocardial infarction.35 ncRNAs are emerging as key modulators of gene regulatory networks. However, little is known about their roles in the myocardium during the adaptive response to hypoxic stress. We have systematically identified the major forms of regulatory ncRNAs found within a cell (ie, lncRNA, miRNAs, and eRNAs). First, our data allowed us to identify differentially expressed pri-miRNA transcripts during myocardial ischemia. We identified most of the classical miRNAs found to be associated with myocardial ischemia/infarction, such as MIR1, MIR21, MIR23, MIR24, MIR27, MIR133a, MIR155, MIR181a, MIR208b, and MIR499.4 However, we also identified novel potential candidates, such as LET7a, MIR99a, MIR103b, MIR148b, and MIR365. Analysis of the mature miRNA levels demonstrated that several miRNAs exhibited no repression despite the effect on transcriptional level. This is consistent with the high stability of miRNAs, where stable miRNA pools might take several days to change.36 This was supported by the observation that downregulated pri-miRNAs were repressed in human myocardium 1 week after infarction. Interestingly, however, 40% of the differentially regulated genes are mostly regulated at the level of initiation or rate of transcription seen as global change in GRO-Seq signal. The release of the polymerase into productive elongation is mediated by positive transcription elongation factor b.
miRNAs belong to the exceptional group of miRNAs with fast turnover⁶⁶ (data not shown), suggesting that transcriptional regulation could play a major role in replenishing degraded miRNAs during ischemia.

In addition, we demonstrate that many of the miRNA target genes are regulated on both transcriptional and post-transcriptional levels, allowing more robust control over gene expression. An example of this was provided by VCL, which is targeted by 16 different miRNAs and further repressed at post-transcriptional level. VCL has also been strongly associated with cardiomyopathy,³⁷,³⁸ suggesting that it may play an important role in cardioprotection. Interestingly, there is growing evidence showing miRNA-mediated epigenetic silencing in the nucleus and, accordingly, it has been reported that human promoters are enriched for miRNA seed matches.¹⁶,³⁹ Preliminary miRNA-binding site predictions within the VCL promoter does identify several of the ischemia-regulated miRNAs, suggesting that miRNAs could participate in transcriptional regulation during oxygen deprivation. Further studies are needed to prove the functionality of these sites.

In addition to miRNAs, GRO-Seq also allowed us to study the alterations in the expression of eRNAs and lncRNAs during an ischemic insult. First, we identify novel antisense RNAs adjacent to and overlapping with several cardiac transcription factors, including GATA4, GATA6, and KLF6. This provides a possible feedback loop where the transcription factors themselves are regulated by ncRNAs. It still remains to be studied whether the expression of lncRNAs is a pre-requisite for the expression of these cardioprotective transcription factors as has been shown for many lncRNAs with enhancer-like functions.⁴⁰ Enhancers contain the majority of the binding sites for stress-inducible transcription factors providing them a central role in driving cell type–specific transcriptional responses.⁴¹ Our data suggest that KLFs are the main regulators of enhancer activity.

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Figure 7. Characterization of bidirectional enhancer RNA transcription in response to ischemic insult. A, Distribution of global run-on sequencing (GRO-Seq) tags around pig myocardial enhancers. B, Correlation of enhancer (e)RNA expression with the nearby coding gene expression showing enhancers located within 10 kb of the closest gene. Pearson correlation values (r) are also shown. C, Sequence motifs associated with differentially regulated eRNAs. D, Cumulative GRO-Seq signal around transcription factor motifs found enriched in (C).
after an acute ischemic event. KLFs belong to the zinc finger family of transcription factors and consist of 17 members that have been shown to play key roles in cellular growth and development. Of these members, KLF4 has been shown to be induced in astrocytes after an ischemic injury, whereas KLF2 protects against ischemic stroke, and KLF10 promotes neurovascularization in response to hindlimb ischemia. Our data demonstrate that the expression of KLF6 and KLF10 is induced on myocardial ischemia whereas that of KLF12 is repressed. We hypothesize that the changes in the overall balance of different KLFs could drive the other transcriptional changes during ischemia. Interestingly, we also demonstrate that most enhancers categorized by specific TF motifs exhibit a global induction of eRNA expression on ischemia with the exception of MEF2C, which is associated with repression of enhancer activity. MEF2 transcription factors play important roles in cardiac development and adaptation to a wide array of physiological and pathological signals by controlling the balance between chromatin acetylation and deacetylation. Previous studies have suggested that interaction of MEF2 with class II histone deacetylases could, through recruitment of corepressors or SUMOylation machinery, repress the activity of MEF2 providing 1 plausible mechanism for enhancer repression. Because histone deacetylase inhibitors have recently surged as potent agents to blunt ischemia/reperfusion injury, it would be interesting to study how much of that is attributed by the derepression of MEF2-dependent transcription.

In conclusion, here we show the immediate transcriptional responses to myocardial ischemia in a clinically relevant large animal model of AMI. For the first time, GRO-Seq was used to detect acute transcriptional changes in response to ischemia in vivo. As many of the detected transcriptional changes are well in-line with the previously published data, we confirm that our porcine model of myocardial ischemia is clinically relevant and able to simulate human myocardial infarction. We identified a clear gradient of transcriptional gene induction or repression accompanied by corresponding changes on protein level when moving from healthy areas toward ischemic zone. We showed that target genes for many miRNAs are regulated on transcriptional and post-transcriptional levels and identified KLFs as the possible main regulators of enhancer activity during myocardial ischemia. Taken together, these findings provide novel insights into the gene regulatory mechanisms that control the responses to myocardial ischemia, and improved understanding of these mechanisms could help to identify new treatment strategies for myocardial ischemia and its complications.

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Disclosures
None.

References
There is increasing evidence that noncoding RNAs (ncRNAs) play important roles in the pathogenesis of myocardial infarction. Here, we used global run-on sequencing to characterize a wide spectrum of regulatory ncRNAs, including primary microRNAs, long ncRNAs, and enhancer RNAs genome wide in a large animal model of acute myocardial ischemia. We demonstrated good correlation between primary and mature micro-RNAs levels, suggesting that transcriptional regulation of myocardial ischemia by extending analysis to a large set of regulatory ncRNAs. The information gained here could be used to recognize factors responsible for (mal)adaptive responses to hypoxia and identify new treatment strategies targeting ncRNAs.

CLINICAL PERSPECTIVE

There is increasing evidence that noncoding RNAs (ncRNAs) play important roles in the pathogenesis of myocardial infarction. Here, we used global run-on sequencing to characterize a wide spectrum of regulatory ncRNAs, including primary micro-RNAs, long ncRNAs, and enhancer RNAs genome wide in a large animal model of acute myocardial ischemia. We identified hundreds of ncRNAs with a clear gradient of expression when moving from healthy to border and ischemic zone. We demonstrated good correlation between primary and mature micro-RNAs levels, suggesting that transcriptional regulation plays an important role in guiding changes in micro-RNA levels in response to ischemia. Furthermore, study of several arrhythmia-related genes suggests that transcriptional regulation and post-transcriptional controls act together to regulate gene expression. Finally, we identified several transcription factor families as possible main regulators of enhancer activity during myocardial ischemia and novel IncRNAs close to these factors. Our data provide a novel insight into the pathogenesis of myocardial ischemia by extending analysis to a large set of regulatory ncRNAs. The information gained here could be used to recognize factors responsible for (mal)adaptive responses to hypoxia and identify new treatment strategies targeting ncRNAs.
Genome-Wide Dynamics of Nascent Noncoding RNA Transcription in Porcine Heart After Myocardial Infarction

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