Long non-coding RNAs in heart failure: a promising future with much to learn

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El Azzouzi and colleagues (1) recently published a perspective describing our work, in which we characterised the murine long noncoding RNA (lncRNA) transcriptome post myocardial infarction (MI) (2). This perspective nicely summarised key aspects of our work. However, we would like to respond with some additional comments to clarify and expand important points resulting from their perspective and our work.

Considering the emerging roles for lncRNAs in dictating the gene regulatory networks underpinning developmental and disease processes (3,4), we set out to characterise this landscape within the infarcted mouse heart (2). We utilised an extremely deep RNA sequencing approach coupled with *de novo* transcript reconstruction to comprehensively profile and discover novel lncRNAs. As discussed, we identified approximately 1,500 previously unannotated lncRNAs, with a significant fraction being differentially expressed post stress. Importantly, through integrating publicly available transcriptomic and epigenomic datasets, we demonstrated that our newly discovered lncRNAs exhibited some unique functional and regulatory characteristics. Firstly, they were significantly more enriched in the heart as compared to protein coding genes (PCGs) or previously annotated lncRNAs. Much of this increased heart specificity is likely as a consequence of their functional genomic loci of origin that are active cardiac enhancer regions. Enhancers are important distally acting cis regulatory sequences (5-7). They are considered the key information processing units within the genome integrating temporal and spatial cues to direct correct context-specific gene expression patterns. Thus, we found that the novel lncRNAs that were modulated post-MI were significantly more associated with active cardiac enhancer sequences, therefore implicating the enhancer landscape and their associated lncRNAs, in the transcriptional reprogramming that underpins pathological remodelling. Finally, and importantly from a translational and therapeutic perspective, we identified hundreds of predicted human orthologs and validated their expression in human samples of a pathological nature (8).

Despite the insightful perspective by El Azzouzi *et al.* (1), we would like to emphasise and clarify a few points. Firstly, what was somewhat unique and surprising during our initial analysis was the sheer number of novel lncRNAs we were able to discover. This, as we have discussed in depth elsewhere (9) and would like to emphasise here, is as a direct consequence of the RNA sequencing depth. We would strongly argue based on our previous experience, that sequencing to a depth of at least 300 million paired-end reads is essential for the identification of novel tissue- and context-specific lncRNAs. With this sequencing depth, we were able to identify the 1,500 novel lncRNAs described in this study but also 2,500 novel lncRNAs in mouse embryonic stem cells (ESC) differentiating towards cardiomyocytes (10). To support our argument, we performed a computational simulation, in which we counted how many novel lncRNAs would have been discovered at different sequencing depths. We found that if we would have sequenced to the widely used depth of 50 million paired-end reads, we would have not detected approximately 50% to 60% of novel lncRNAs in both our studies (9). Strikingly, one should consider that the novel lncRNAs that required the greatest depth of sequencing for discovery corresponded to those exhibiting the most interesting regulatory and functional characteristics, in particular heart-enriched lncRNAs associated with cardiac developmental and functional roles. Those lncRNAs that

were discovered at the shallower sequence depth were typically already annotated housekeeping like and non-tissue enriched lncRNAs.

El Azzouzi *et al*. (1) highlighted the cardiac-enriched nature of our novel lncRNAs. Despite validating a number of them via qPCR, it is important to note that this conclusion was primarily based on the integration of publicly available tissue-specific RNA-seq datasets. By using ENCODE data, we quantified the expression of every transcript in the heart and 17 non-cardiac tissues (2,9). Heart specificity was then determined for each transcript by calculating the expression in the heart versus the mean expression in all 17 non-cardiac tissues, generating a specificity score for each transcript (9). This approach is powerful and has been successfully used for lncRNAs discovered in a number of cell types and tissues demonstrating that newly discovered lncRNAs are typically more tissue-enriched *vs*. PCGs and also miRNAs (11). This is of important therapeutic potential, as targeting cell- and tissue-restricted lncRNAs could increase the specificity of future therapies and reduce off-target side effects. These observations also support the notion that lncRNAs could represent highly specific biomarkers for pathological processes (11,12). Indeed, a number of recent studies are beginning to validate this hypothesis. Along the same line, we demonstrated that cardiac lncRNAs expression is extremely well correlated with electrocardiographic traits. It is important to emphasise here that this correlation was executed in mouse and not in human patients as mentioned by El Azzouzi *et al*. (1). Comparable observations have however been made in human patients (11,12), supporting our work in mice. Furthermore, a number of recent studies have demonstrated the presence of lncRNAs circulating in the plasma (12). The detection of cardiac-enriched lncRNAs in the plasma therefore warrants interrogation and promises to be an exciting future application of our work with respect to post infarction remodelling and heart failure.

Despite the relative ease in discovering lncRNAs implicated in cardiovascular development and disease, elucidating their functions remains a major challenge. We applied a novel approach to infer functions for lncRNAs based on chromatin state transitions during the differentiation of ESCs to cardiomyocytes (2,9). We believe this approach represents a relatively simple method to begin to prioritise cardiovascular lncRNAs for functional characterisation. The analysis is based on the observation that PCGs in differentiating cells cannot be assigned to a particular function based on their expression kinetics alone. However, Wamstad *et al*. (13) found using genomewide epigenomic profiling that PCGs co-expressed during cardiac differentiation can be functionally grouped based on distinct chromatin state transitions at their promoters. More specifically, they concluded that sub-groups of co-expressed PCGs, clustered based on unique chromatin state transitions, were involved in highly specific and distinct biological processes pertinent to cardiac development and function. These biological processes included cardiac signalling, metabolism, development and muscle contraction. We therefore surmised that novel lncRNAs sharing these unique chromatin state patterns at their promoters were likely to be involved in parallel biological processes. This approach therefore provides a very powerful and unbiased proxy to infer functions for novel lncRNAs. Supporting this, we found that heart-enriched novel lncRNAs were preferentially associated with clusters linked to fundamental cardiac biological processes including cardiac contraction and development. Finally, we also used this approach to predict targets of novel lncRNAs based on shared chromatin state patterns. For example, we demonstrated that Novlnc6 was able to regulate Bmp10, which shared the same chromatin patterns during differentiation. This resulted in a specific impact on the key core cardiac transcription factor, Nkx2-5, a known target of Bmp10 (2).

To conclude, our work, in addition to a number of other studies characterising the long noncoding transcriptome in disease (14,15), opens a new era of vast therapeutic and diagnostic potential. This highly integrated layer of noncoding RNAs exhibits interactions with components of various effector complexes to dictate the activity of developmental and pathological cardiac gene regulatory networks. Our data clearly demonstrates that pathological states within the heart are intrinsically linked to the dynamics of lncRNA expression. In the future, it will be of critical importance that investigators discovering novel cardiovascular lncRNAs leverage both computational and experimental approaches to functionally characterise the landscape of lncRNAs within the heart (9). In particular, one should focus on deciphering the mechanisms and language through which lncRNAs interact with their respective protein, RNA or DNA partners. These approaches should illuminate our understanding of these very exciting therapeutic and diagnostic molecular targets.

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Footnote

Provenance: This is a Guest Letter to the Editor commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).
Conflicts of Interest: The authors have filed a patent about the diagnostic and therapeutic use of several heart enriched lncRNAs.

Response to: El Azzouzi H, Doevendans PA, Sluijter JP. Long non-coding RNAs in heart failure: an obvious lnc. Ann Transl Med 2016;4:182.

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