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RAPID COMMUNICATION

Downregulated nuclear lncRNA NRON inhibits SHP2/Wnt/β-catenin signaling and cardiomyocyte differentiation during the development of Tetralogy of Fallot

Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart disease and the incidence of late cardiac death in long-term survivors continues to increase.[1] So, there is an urgent need to explore the etiology and pathogenesis of TOF. The precise cause of TOF is currently unclear, and exploration of the pathogenesis has focused increasingly in recent years on the roles of noncoding gene products, especially long noncoding RNAs (lncRNAs). The lncRNA *NRON* is localized at the 9q33.3 revered strand. The related literature has revealed that its transcript is enriched in human cardiac muscle[2] and *NRON* overexpression has been reported to reverse epithelial-to-mesenchymal transition (EMT)[3] which is known to contribute to valve development in the heart. Based on these findings, we hypothesized that abnormal expression of NRON was probably relevant to congenital heart defects. This study investigated the role of lncRNA *NRON* in the nucleus and its correlation with cardiac development and TOF.

To demonstrate the correlation between *NRON* and cardiac development, the temporal expression profile of *NRON* was examined in C57BL/6J mice from the embryonic day 9.5 (E9.5) to adulthood. We found that the transcription of *NRON* started at E10.5, followed by an increasing trend from E10.5 to P7, and then decreased in adulthood (Fig. 1A; Fig. S1A). Then eight embryonic hearts from human normal fetuses and eight from fetuses with TOF at an average gestational age of 20 weeks were collected and the results showed that the expression levels of *NRON* were significantly downregulated in the TOF group ($P < 0.0001$) (Fig. 1B). These findings raise the possibility either that the lncRNA *NRON* may be involved in the regulation of cardiac development or that abnormal expression of *NRON* may be one of the factors affecting the occurrence and development of TOF.

To confirm how LncRNA NRON is involved in heart development, we used a model in which hESCs could be induced to differentiate into cardiomyocytes *in vitro* to observe the role of NRON. We examined the expression of *NRON* during hESC differentiation and found that *NRON* expression increased significantly, reaching a peak at 6 days and then gradually decreasing until it remained at a relatively low level at 30 days when cardiomyocytes matured (Fig. S2A). Given this trend, an episomal vector-based CRISPR/Cas9 system[4] was applied to construct an NRON-knockout hESC line (NRON-KO hESCs) and an empty-vector cell line (empty vector hESCs) for differentiation experiments (Fig. S2B). Detailed methods were described in the supplementary materials. We collected cells at different differentiation days (0, 2, 4, 6, 8, 15) and detected the levels of markers in two groups at different differentiation stages.

The pluripotency markers *OCT4* and *NANOG* showed no differences in expression between the two undifferentiated groups (Fig. S2D). Beginning at 2 days, the expression of the early mesoderm genes *T* and *MIXL1* in the NRON-KO hESCs was significantly suppressed (Fig. S2E, F). Core transcription factors (*NKX2.5*, *MEF2C*, *GATA4*) are important regulators of cardiomyocyte development and their transcript levels were significantly decreased in NRON-KO hESCs at 4, 6, and 8 days (Fig. S2G–I). As a result, the expression of cardiomyocyte markers almost disappeared in NRON knockout group at the end of differentiation (Fig. S2J).

The first beating cell clusters were observed at 8 days in the empty vector hESCs, and almost all cells were beating after 12 days. However, we did not observe this phenomenon in NRON-KO hESCs (Fig. S2K). To further demonstrate

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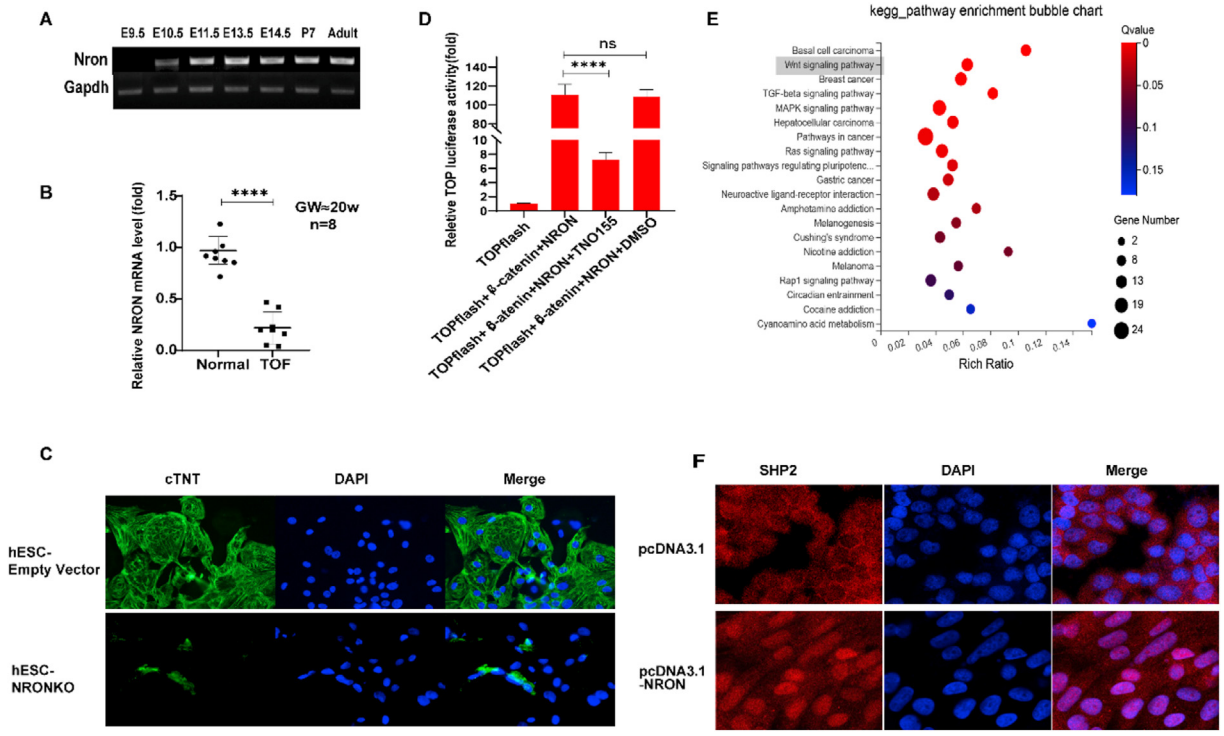

Figure 1 The correlation between *NRON* and cardiac development. **(A)** RT-PCR confirmed the temporal expression profile of *Nron* in C57BL/6J mice. **(B)** The lncRNA *NRON* was significantly downregulated in embryonic hearts of the TOF group compared with that in the normal heart group, detected by RT-qPCR. Values are expressed as mean ± SEM, $n = 8$, ****$P < 0.0001$. GW, gestational week. **(C)** Immunofluorescence analysis at 15 days. Blue fluorescence indicates the DAPI-stained nuclei, and green fluorescence was from cTNT. hESC, human embryonic stem cell H9. CMs, cardiomyocytes. hESC-Empty Vector, empty vector hESCs. hESC-NRONKO, NRON-knockout hESCs. **(D)** Dual-luciferase reporter assay. TNO155, an SHP2 inhibitor, reduced the relative TOPflash activity elevated by *NRON*. DMSO served as a control group. The data presented are the mean ± SEM of at least three independent experiments. *ns*, nonsense. ****$P < 0.0001$. **(E)** The bubble plots showing KEGG pathway enrichment data for genes that were downregulated in the NRON-KO group. The *X*-axis is the rich ratio and the *Y*-axis is the enrichment pathway. The blue color with a higher saliency indicates a higher enrichment significance, while the red with a higher saliency indicates a lower enrichment significance. **(F)** AC16 cells were infected with pcDNA3.1 and pcDNA3.1-NRON. After 24 h, two groups of cells were collected for laser confocal observation of the cellular distribution of SHP2. Magnification, 60 × . DAPI, blue. SHP2, red. AC16, human cardiomyocyte-like cells.

the presence of cardiomyocytes, immunofluorescence analysis was performed, which verified that few cTNT$^+$ cells were present among NRON-KO hESC (Fig. 1C). All these results suggest that low expression of *NRON* prevents hESCs from differentiating into functional cardiomyocytes. The severely reduced expression of mesodermal markers in NRON-KO hESCs suggests that hESC differentiation to the mesoderm is blocked, which may be the initiating factor leading to the failure of cardiomyocyte differentiation.

Abnormal expression of *NRON* can affect the differentiation of hESCs into cardiomyocytes, and our differentiation approach *in vitro*[5] is primarily involved in triggering the canonical Wnt signaling pathway. Therefore, we hypothesized that the lncRNA NRON promoted cardiomyocyte differentiation by activating the Wnt/β-catenin signaling pathway. To test this hypothesis, we first examined the expression of Wnt target genes, including *cyclinD1*, *Myc*, and *Axin2*. As shown in Figure S3A, the expression of these three genes was significantly decreased in the NRON-KO hESC group, while there was no difference in the β-catenin expression level. Subsequently, a dual-luciferase reporter assay showed that the relative TOPflash activity was increased by 2 folds in cells simultaneously transfected with pcDNA3.1-NRON and β-catenin, compared to cells transfected with β-catenin only (Fig. S3B). TOPflash activity could also be seen even in cells transfected with pcDNA3.1-NRON. These results suggest that the lncRNA *NRON* can activate the Wnt/β-catenin signaling pathway. At the same time, the cellular RNAs from NRON-KO hESCs and empty vector hESCs were subjected to RNA-sequencing analysis and the result suggests that downregulated genes in NRON-KO hESCs were enriched in the WNT signaling pathway (Fig. 1E).

To explore how *NRON* activates the WNT signaling pathway, we first performed *NRON* subcellular localization analysis by subcellular fractionation assay, which showed that more than 80% of *NRON* was in the nucleus (Fig. S1B, C). Then we used RNA pulldown assays followed by mass spectrometry (MS) to identify NRON-binding proteins in the nucleus. MS analysis showed that eight proteins were associated with cardiac development. Among them, only

SHP2 was correlated with the activation of the WNT signaling pathway. So, we selected SHP2 as the target protein and validated the MS results by western blot (WB). The results support that SHP2 specifically binds to *NRON* (Fig. S3C).

Since SHP2 can activate the Wnt/β-catenin signaling pathway by promoting the binding of parafibromin to β-catenin, we speculated that *NRON* might activate the Wnt signaling pathway through SHP2. As expected, we acquired consensus results that the relative TOPflash luciferase activity was sharply decreased by more than ten times upon the use of TNO155, a potent inhibitor of wild-type SHP2 (Fig. 1D).

We further explored how *NRON* interacts with SHP2. WB analysis of SHP2 showed no expression difference between empty vector hESCs and NRON-KO hESCs (Fig. S3D), indicating that SHP2 was not the target protein of *NRON*, but probably a partner. SHP2 can be distributed to both the cytoplasm and nucleus at low cell density but is excluded from the nucleus at high cell density. So, we examined the effect of *NRON* on the location of SHP2 in high-density AC16 cells by laser confocal microscopy. As shown, SHP2 accumulated mainly in the nucleus in cells transfected with pcDNA3.1-NRON, yet it accumulated in the cytoplasm in cells transfected with the empty vector (Fig. 1F). Taken together, these findings support that *NRON* activates the Wnt/β-catenin signaling pathway probably by promoting SHP2 nuclear accumulation.

In summary, during cardiac development in TOF, *NRON* expresses at very low levels; such low expression may cause most SHP2 to shift to the cytoplasm and fail to activate the Wnt/β-catenin signaling pathway in the nucleus, thus affecting the normal differentiation of cardiomyocytes. However, we do not know which parts of SHP2 and *NRON* interact with each other or what other proteins might be involved in the complex formed by these molecules. We will address these issues in the future.

Author contributions

H.Z. contributed to study design, data analysis, and manuscript drafting. L.L. performed experiments and revised the manuscript. C.L. and H.D.L. collected the clinical samples and information and performed experiments. H.Y.W. and J.T. provided funding and directed the project. All authors read and approved the final version of the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.10.010.

References

1. Karamlou T, McCrindle BW, Williams WG. Surgery insight: late complications following repair of tetralogy of Fallot and related surgical strategies for management. *Nat Clin Pract Cardiovasc Med*. 2006;3(11):611–622.
2. Sharma S, Findlay GM, Bandukwala HS, et al. Dephosphorylation of the nuclear factor of activated T cells (NFAT) transcription factor is regulated by an RNA-protein scaffold complex. *Proc Natl Acad Sci U S A*. 2011;108(28):11381–11386.
3. Yao Z, Xiong Z, Li R, et al. Long non-coding RNA NRON is downregulated in HCC and suppresses tumour cell proliferation and metastasis. *Biomed Pharmacother*. 2018;104:102–109.
4. Xie Y, Wang D, Lan F, et al. An episomal vector-based CRISPR/Cas9 system for highly efficient gene knockout in human pluripotent stem cells. *Sci Rep*. 2017;7(1):2320.
5. Lian X, Hsiao C, Wilson G, et al. Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proc Natl Acad Sci U S A*. 2012;109(27):E1848–E1857.

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