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Short Communication

Epigenomic profiling identifies the role of Nr5a2 and CYP1B1 in hepatocellular carcinoma stemness maintenance

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Cancer stem cells (CSCs) are largely responsible for the formation of tumor heterogeneity and tumor's resistance to traditional treatments such as chemotherapy due to its self-renew capability and multi-lineage differentiation potential [1]. However, it remains elusive how the CSCs maintain their stemness, thus restricting the development of therapeutic treatments specifically targeting the CSCs. Accumulating evidence has suggested that the genetic and epigenetic changes are responsible for the acquisition of stemness and CSC genesis in hepatocellular carcinoma (HCC) [2], which is a leading cause of cancer-related deaths worldwide [3]. In this study, we integrated Hi-C, ATAC-seq and RNA-seq data together with public ChIP-seq sources, to establish the relationship between the epigenetic modulation and transcriptional regulation of the stemness maintenance of HCC CSCs. This multi-omics method shed lights on the utility of epigenomic profiling to uncover the gene regulation mechanism of CSCs and could efficiently identify promising drug targets for HCC targeted therapy.

Intratumoral and intertumoral heterogeneity have been previously observed in tumor samples obtained from different patients [4]. Previously, our group used the "Side Population (SP)" assay to isolate CSCs from HCC cell lines and fresh clinical HCC tissue samples, and we found that both of them harbored similar CSCs properties [5]. Based on these observations, we reasoned that an in vitro cell culture model of HCC CSCs would greatly facilitate our study for the molecular mechanism of CSCs stemness maintenance. In this study, Huh7 cell line was used as a model system to enrich for CSCs through the SP assay. The percentage of SP cells in Huh7 was 1.383 ± 0.079% (n = 12) (Fig. S1a,b online). To test whether the SP cells harbor CSCs properties, RT-qPCR analysis was conducted to quantify the relative expression of stem cell-related genes, including *ABCG2*, *CD133*, *EpCAM*, *CD13*, *KLF4*, *OCT3*, *SALL4* and *ALDH*. Except for *SALL4*, all of these genes have significantly higher expression in SP cells as compared to NSP cells (n = 5) (Fig. S1c online). Cell proliferation assay was implemented to examine whether the SP cells have similar growth property to typical CSCs. Equal numbers of unsorted cells and sorted NSP & SP cells were cultured and stained, and the formazan absorbance at 450 nm was measured at different time points. The SP cells presented an almost two-fold higher proliferation rate than the NSP cells in culture (n = 5, P < 0.001) (Fig. S1d online). The SP cells also had a higher rate of self-renewal (n = 4, P < 0.01), as indicated by the spheroid colony formation assay (Fig. S1e,f online). Specifically, the majority of the NSP cells were dead after culturing in serum-free medium after 9 days, while both the SP cells and unsorted Huh7 cells formed tumorspheres, which were defined as diameter >50 μm. The number and size of tumorspheres were substantially bigger in the SP cell cultures than in the NSP and unsorted cell cultures. In addition, *in vitro* transwell invasion assay also showed that the SP cells displayed significantly stronger invasion capability than the NSP cells (n = 5, P < 0.001) (Fig. S1g,h online). Altogether, the results suggested that the SP cells enriched from Huh7 cell culture possessed key CSCs properties, including preferential expression of stem cell-related genes, higher proliferation capacity, increased colony-formation ability and stronger invasion ability. We thus reasoned that the sorted SP cells from Huh7 could be used as representative HCC CSCs for the subsequent studies.

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To elucidate the gene expression pattern and regulatory gene pathways in SP cells, RNA-seq analysis on sorted SP and NSP cells was performed with well replicated two biological repeats (Fig. S2a online). Differential expression analysis showed that 783 genes were significantly up-regulated and 273 genes were down-regulated (|$\log_2$fold change| > 1; P_{adjust} < 0.01) in the SP cells as compared to the NSP cells (Fig. 1**a**). GO term and KEGG annotation indicated that the significantly up-regulated genes in SP cells

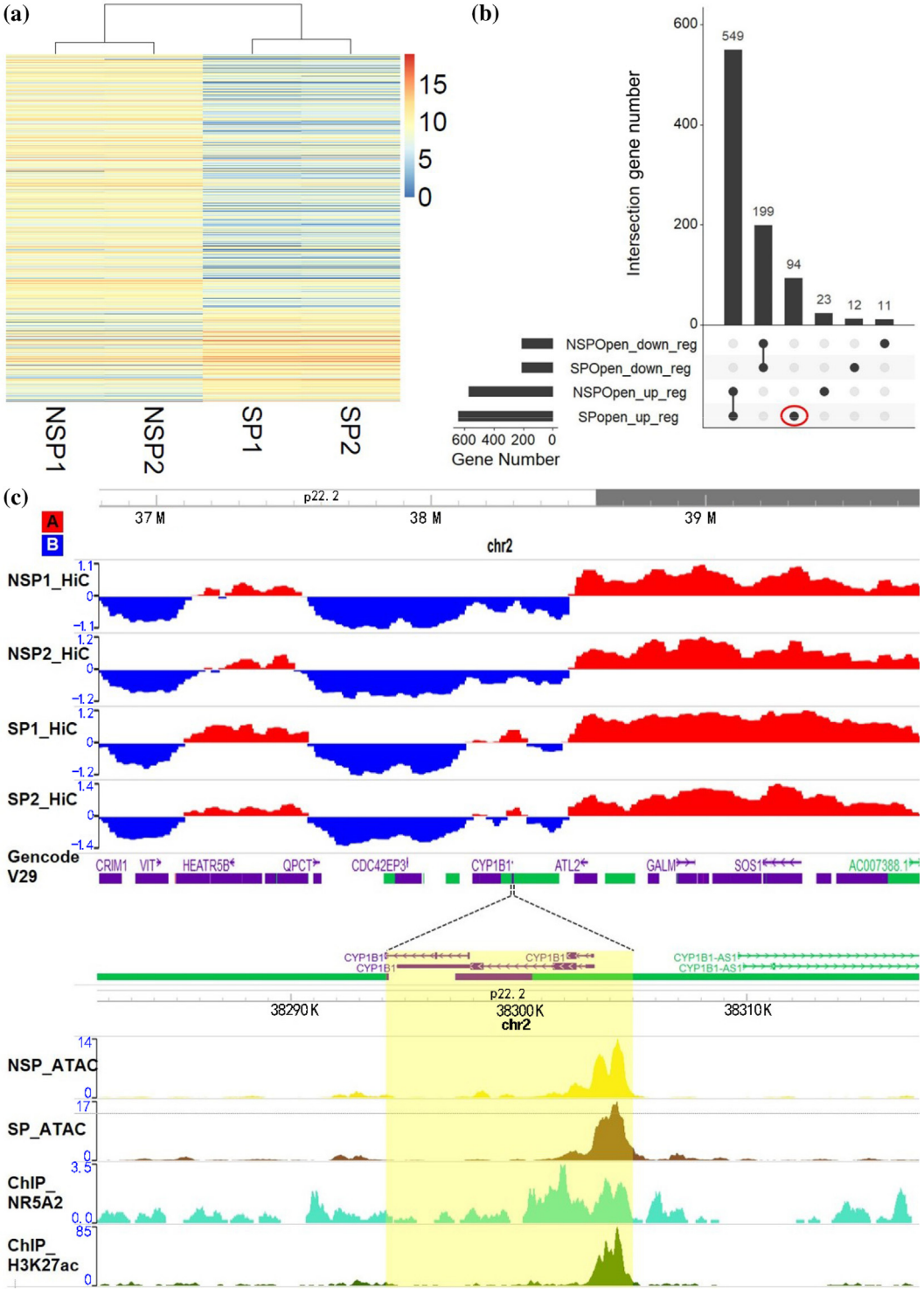

Fig. 1. (a) The heatmap of differentially expressed genes from two sets of independent biological replicates (SP & NSP) was shown. Yellow indicates up-regulated genes and blue indicates down-regulated genes. (b) 94 up-regulated genes located in the regions that switched from the "closed" chromatin state in NSP cells into "open" state in the SP cells. (c) There is a chromatin state and compartment switch in CYP1B1 located region. Nr5a2 was shown to directly bind to the promoter of *CYP1B1* and increase the expression of *CYP1B1* to orchestrate stemness maintenance of CSCs in HCC. All of the visualization of reads were presented on the WashU Epigenome Browser. Red indicates compartment A regions and blue indicates compartment B regions.

were mostly involved in drug metabolism, lipid metabolic process, and cell–cell junction organization (Fig. S2b,c online). Notably, the *cytochromes P450 (CYP)* genes, a family of genes responsible for drug metabolism and other xenobiotics, fatty acid, cholesterol and bile acid metabolism [6], were all up-regulated in the SP cells, as confirmed by the RNA-seq and RT-qPCR analyses. *MICAL1* and *ANXA1* that promote cancer cell proliferation and invasion were also up-regulated in the SP cells as compared to the NSP cells (Table S1 and Fig. S3 online). Altogether, our transcriptome analyses showed that the SP cells maintained a distinctive stemness gene expression signature while exhibited activation of unique oncogenic pathways.

To further elucidate the epigenomic regulatory mechanisms in HCC CSCs, ATAC-seq was performed to map the genome-wide chromatin accessibility and the activation status of epigenomic regulatory elements, such as promoters and enhancers, in the SP cells (Fig. S4a and Table S1 online). Most of the peaks were distributed inside and in the vicinity of the genes (Fig. S4b online). In parallel, we collected public Huh7 ChIP-seq data of histone modifications to call chromatin states (Fig. S5a online) and annotated accessible chromatin with these data to define open enhancers and promoters. Combined with our ATAC-seq results, we found that most accessible chromatin regions were located at gene promoters and enhancers (Fig. S5b online). Substantially more open enhancers were found in the SP cells than in the NSP cells, with 23,781 for the SP cells and 15,907 for the NSP cells, respectively. The ATAC-seq data were integrated with the transcriptome data and 643 up-regulated genes in "open" chromatin regions of the SP cells were identified. The genes were mainly enriched in cell junction organization, lipid metabolic and xenobiotic metabolic processes (Fig. S5c online). 94 of the up-regulated genes were found to switch from the "closed" chromatin state in NSP cells into 'open' state in the SP cells (Fig. 1b). Furthermore, functional enrichment analysis showed that the change of chromatin accessibility landscape in the SP cells had positive correlation with the drug resistance, metabolic dysfunction and high migration capacity of CSCs (Fig. S5d online). To further identify putative TFs binding at the ATAC-seq peaks, motif binding scores of a large set of known TFs were analyzed. A total of 33 TFs with significant binding scores were discovered in the SP and NSP cells (Table S3 online). Notably, the SP cells preferentially harbored motifs of Nr5a2 and Klf13. Nr5a2 is involved in a wide variety of biological processes, including cholesterol and glucose metabolism in the liver, pancreatic development [7], and treatment with the Nr5a2 antagonist (LRA) inhibited the proliferation and colony formation of hepatoblastoma cell lines [8], corroborating the finding that Nr5a2 is involved in orchestrating CSCs properties of the Huh7 SP cells.

Principal component analysis (PCA) of Hi-C data uncovered that the genome is segmented into two types of compartments (A versus B). The switch between A/B compartments is related to transcriptional regulation and cell fate decision [9]. To investigate how the higher-order chromatin structures might regulate gene expression to maintain HCC CSCs stemness, *in situ* Hi-C between independent replicates was performed (Table S4, Fig. S6a,b online). Since the dynamic switching between A and B compartments could permit the dynamic transcriptional regulation in the SP cells, we focused on the significant changes in chromatin compartments with PC1 values ($|\log_2 \text{fold change}| > 1$; $P_{\text{adjust}} < 0.05$), and identified 1732 genes in compartment A and 1261 genes in compartment B. By comparing Hi-C interaction maps of the SP and NSP cells with the transcriptome data and ATAC-seq data, 12 up-regulated candidate genes in the open chromatin state located in the compartment A regions of SP cells were identified (Table S5 online). Among them, *CYP1B1* is most notable and the located region of *CYP1B1* gene is found to switch from "closed" chromatin state in compartment B of NSP cells to "open" chromatin state in compartment A of SP cells (Fig. 1c). Previous studies indicated that CYP1B1 is expressed at higher levels in malignant tumors compared to normal tissues and has been proposed as an early stage tumor marker [10]. In addition, Nr5a2 MNChIP-seq data in human ESCs showed that there is a binding site of Nr5a2 in the promoter of *CYP1B1*.

Elucidating how the CSCs maintain their stemness through epigenetic regulation could open up new perspectives for developing therapeutic treatment to eliminate CSCs in the tumor. In this study, epigenomic profiling was used to systematically examine the epigenomic regulatory networks in HCC CSCs, and found a potential role for Nr5a2 in the stemness maintenance through up-regulating the expression of *CYP1B1*. The systematic epigenomic profiling provided a solid framework for a better understanding of the relationship between epigenetic modulation and transcriptional regulation in the stemness maintenance of CSCs. The analyses reported and related experimental confirmation suggested that our strategy could efficiently promote the identification of therapeutic targets toward HCC CSCs.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2019.06.017.

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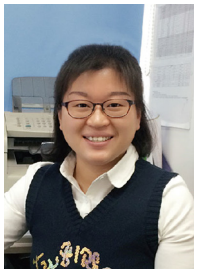

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