



Super-enhancer-mediated core regulatory circuitry in human cancer

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ABSTRACT

Super-enhancers (SEs) are congregated enhancer clusters with high level of loading of transcription factors (TFs), cofactors and epigenetic modifications. Through direct co-occupancy at their own SEs as well as each other's, a small set of so called "master" TFs form interconnected core regulatory circuitry (CRCs) to orchestrate transcriptional programs in both normal and malignant cells. These master TFs can be predicted mathematically using epigenomic methods. In this Review, we summarize the identification of SEs and CRCs in cancer cells, the mechanisms by which master TFs and SEs cooperatively regulate cancer-type-specific expression programs, and the cancer-type- and subtype-specificity of CRC and the significance in cancer biology.

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1. Introduction

Deciphering how transcriptional program is orchestrated to maintain cell identity and cell fate is fundamental to understand

normal developmental processes and disease pathogenesis. Cell-type-specific gene transcription is primarily governed by master transcription factors (TFs) and *cis*-regulatory elements. Super-enhancers (SEs), a unique group of *cis*-regulatory elements, are composed of multiple of adjacent enhancers, which are occupied by a high density of cell-type-specific master TFs, epigenetic regulators and coactivators, driving the expression of genes determining cell identity and cell-type-specific functions [1–5].

Mechanistically, SEs require the cooperation with TFs to control gene expression programs and the activity of SEs is affected by the change of TF enrichment. On the other hand, expression of master TFs that bind SEs is often regulated by the activity of SEs, indicating

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the existence of a positive feedback regulation between SEs and master TFs. Indeed, core regulatory circuitry (CRC) has been established in both normal (e.g., embryonic stem cells (ESCs)) and malignant cells [5–16]. In such regulatory circuitries, master TFs form an auto-regulated loop with SEs of themselves, as well as an interconnected loop with other master TFs and their SEs, resulting high expression levels of each TF within CRC. Moreover, multiple TFs co-occupy the same SE constituents in a cooperative manner, which increases the redundancy in transcriptional activation. As a result, the CRC structure is relatively stable and robust. Nevertheless, considering the prominent role of CRC in cancer, it serves as an attractive target [8,11,13,17]. Indeed, by perturbing the activity of various transcriptional regulatory components, including RNA polymerase II-dependent elongation, chromatin architecture and SE activity, transcriptional and epigenetic inhibitors such as CDK7 inhibitor, BET and HDAC inhibitors have shown encouraging anti-neoplastic potential in (pre)clinical trials in various types of malignancies [3,11,13,17–26].

Here, we review the strategy and methodology for the identification of SEs and CRC, highlight the cancer-type specificity of SEs and CRC, and discuss questions yet to be solved and possible developments on targeting CRC for the treatment of human cancers.

1.1. Super-Enhancers (SEs)

Enhancer is a type of DNA *cis*-regulatory element, which is bound by TFs to promote gene expression. As large clusters of enhancers spanning tens of kilobases of genomic region, super-enhancers (SEs) govern cell-type-specific gene expression and contribute to cancer development through direct (often long-range) interaction with target promoters [1–2,5]. The term “super-enhancer” was first coined by Chen and colleagues in 2004 to define one functional enhancer of homologous region-3 of *Bombyx mori* nucleopolyhedrovirus [27]. The concept of SEs has been greatly promoted by Young’s group based on the crucial role of SEs in the control of cell identity and disease [1,3,5,28].

In the earlier studies [5], chromatin immunoprecipitation sequencing (ChIP-seq) of Oct4, Sox2, Nanog (OSN) was generated to annotate regulatory DNA elements in murine ESCs which identified a total of 8794 enhancers. Interestingly, 231 of these enhancers appeared to be outliers: they exhibited as clusters of closely-distributed enhancers which covered very large DNA regions (an order of magnitude longer than other regular enhancers in median length), and they were termed “SE”. In addition, these “outliers” showed prominently high levels of occupancy of master TFs, transcriptional coactivators such as Mediator (Med1), active histone modifications H3K27ac and H3K4me1, as well as higher DNase I hypersensitivity (by at least an order of magnitude higher than other enhancers for Med1, H3K27ac and H3K4me1 signals). Importantly, when Young’s group manually inspected the 210 genes associated with these 231 SEs, they were impressed that these SE genes contained a large number of factors that had hitherto been implicated in the regulation of ESC identity and stemness, including ESC-specific TFs (OSN), reprogramming facilitating factor (*Klf4* and *Esrrb*), microRNAs such as *miR-290–295*, as well as chromatin modifying enzymes (*Tet1* and *Tet2*). They then went on to characterize other cell types and showed that SEs were also associated with prominent cell-fate defining factors in mouse myotubes, T helper cells, and macrophages, etc. These findings led to the conclusion that SEs are not merely larger in size, but they control an important class of cell-type specific genes. In different types of cancers, SEs control the expression of prominent tumor-promoting genes, such as *MYC* [1,12,29], *TP63* [11,25,30–31], *MYCN* [10,32] and *TAL1* [16,33], and mediate transcription dysregulation of cancer cells.

These results reveal some of the key features of SEs: 1) SEs contain clusters of closely distributed enhancers; 2) SEs are much longer than typical enhancers; 3) SEs show strong enrichment of various TFs, co-factors and active chromatin marks. These characteristics are reflected in the assumptions and implications of methods for SE identification. For example, in the ROSE program, enhancers called by ChIP-seq signals (e.g., H3K27ac) are stitched together if they are within 12.5 kb. Both of the stitched enhancers and solo enhancers are ranked on the basis of enrichment of normalized ChIP-seq signals. In the resultant curve, SEs are defined as those regions with an inflection value ≥ 1 , and the remaining enhancers with an inflection value < 1 are defined as typical-enhancers (TEs) (Fig. 1A).

Clearly, these assumptions and implications of SE definition have their limitations. For example, the robustness and rigor of ROSE is largely affected by both the stitching parameter and extreme SEs. In particular, the inflection value, which is used to define SE/TE, is greatly influenced by extremely large SEs. Indeed, simply removing just a few of the top-ranking SEs can produce a substantial effect on the ranking curve, resulting in the change of classification of many “borderline” SEs. Therefore, the following strategies can be considered to enhance the robustness and reproducibility of SE annotation: 1) using different epigenomic factors to annotate SEs, such as Med1, H3K27ac and BRD4; 2) overlapping SEs from multiple replicate samples; 3) adding a classification of “borderline” SEs by adjusting the inflection value. The “borderline” SEs are to be considered as low-confident SEs.

Additionally, promoter regions can be a source of confounding signals. Active enhancers are often measured by ChIP-seq using the H3K27ac antibody. Since H3K27ac marks both active enhancers and active promoters, SE annotation using this approach may lead to promoter signal being recognized as enhancer. Therefore, it is recommended that SEs are annotated by only considering regulatory elements after removing active promoters, which can be assessed by matched H3K4me3 ChIP-seq profiles. In the absence of such data, one can remove ± 2.0 kb window flanking known transcription start sites (TSS) [1,4]. Clearly, TSS annotation source is an important factor and non-coding genes are suggested to be included.

In comparison with TEs, SEs have stronger activity on transcriptional regulation and are associated with more RNA production. As a result, SE-associated genes have higher expression levels and are more sensitive to the perturbation by transcriptional or epigenetic inhibition relative to TE-associated genes [3,25,32,34–35] (Fig. 1B). Moreover, assay of transposase-accessible chromatin with sequencing (ATAC-seq), a sensitive method to map chromatin accessible regions [36], confirms the high accessibility of SEs [11,13,22,37].

In different types of cancers, several mechanisms of SE-mediated dysregulation have been reported. For example, somatic mutations at the upstream of *TAL1* gene introduce the binding site of MYB to form an SE, leading to the overexpression of *TAL1* oncogene [33]; focal amplification of SEs activates the expression of *MYC* and *KLF5* in T-ALL, acute myeloid leukemia (AML) and squamous cell carcinomas, respectively [29,38–40]; and activation-induced cytidine deaminase (AID)-triggered translocation brings the *c-myc* oncogene to the SE region of *IgH*. This mechanism effectively introduces a *de novo* SE for *c-myc* and results in high-expression of *Myc* oncogene, which promotes B cell lymphomagenesis [41–43]. These observations highlight that genomic and epigenomic alterations in malignant cells activate SEs to contribute to cancer biology. Currently, there are several curated databases providing comprehensive online resources for the identification of SEs, SE-associated genes as well as genetic and epigenetic annotation on SEs,

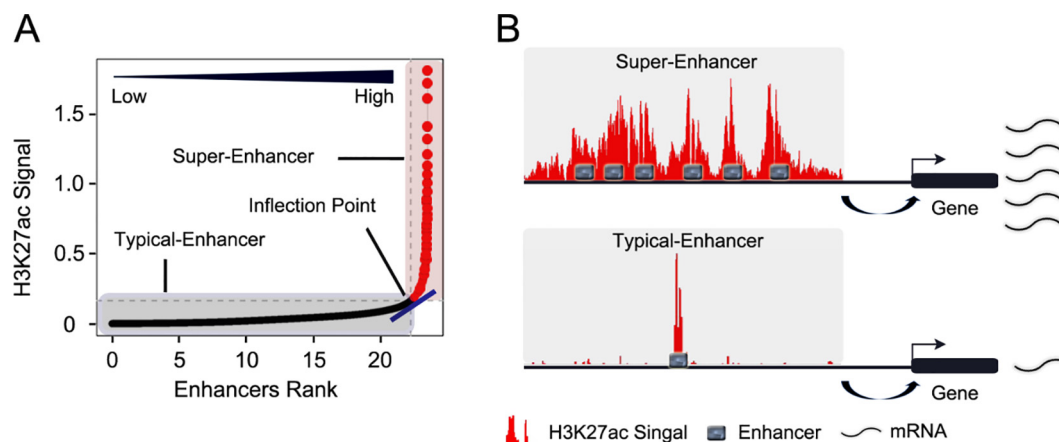


Fig. 1. Defining Super-Enhancers (SEs) and Typical-Enhancers (TEs). (A) Hockey stick plot identifying SEs and TEs based on input-normalized H3K27ac ChIP-seq signals. Enhancer regions are ranked on X axis based on the ChIP-seq signals given on Y axis. SEs are defined as those regions with an inflection value ≥ 1 (Red dots), and the left regions are defined as typical-enhancers (TEs) (highlighted in gray). The “low–high” bar denotes the density of reads in each region. (B) Integrative genomics viewer (IGV) showing the density of ChIP-seq signals (such as H3K27ac) at SE/TE-regulated gene loci. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

including SEdb [44] (<http://www.licpathway.net/sedb/>), SEA [45] (<http://sea.edbc.org/>), dbSUPER [46] (<https://asntech.org/dbsuper/>), and SEanalysis [47] (licpathway.net/SEanalysis/).

1.2. Cancer-Type and -Subtype Specificity of SEs

Representing a major clinical challenge, inter- and intra-tumor heterogeneity is an important field in cancer research. On the basis of biological and/or molecular features, many cancer subtypes have been established with significant implications in clinical management. Importantly, by integrative epigenomic analysis including DNase I hypersensitive sites (DHSs), chromatin accessibility and enhancer profiling by ChIP-seq, cancer-type and -subtype specific enhancers have been identified in a number of samples.

For example, using SE landscapes generated by H3K27ac and BRD4 ChIP-seq data from 28 primary medulloblastoma specimens, subgroup-specific identity of WNT, SHH, Group 3, and Group 4 medulloblastomas was characterized [12]. This study further revealed subgroup-specific transcriptional dependencies and heterogeneity of cellular origins of medulloblastomas. By comparative analysis of H3K27ac ChIP-seq data from Roadmap Epigenomics Consortium with 42 ependymomas tissues, Mack et al. showed subgroup-specific enhancer profiles of ependymomas, and identified SE-associated therapeutic targets and pathways for this rare cancer [48]. In AML, unique regulatory evolution and subtype-specific regulatory network which was associated with specific mutation patterns have been uncovered by comprehensive analysis of chromatin accessibility [49], H3K27ac ChIP-seq [50], and DHSs [51] datasets. In addition, AML epigenomic subtypes showed distinct sensitivity to pharmacologic inhibition. For example, responsiveness to RAR α agonist (SY-1425) depended on the presence of a RAR α SE and high expression of RARA in a subset of AML samples [50]. Similarly, distinct chromatin responses to HDAC inhibitors have been observed in cutaneous T cell lymphoma (CTCL) leukemia, host and normal CD4 + T cells by mapping opening chromatin SEs from 111 human CTCL patients and normal individuals [22]. The cancer-subtype specificity of SEs has also been observed in other tumor types, including rhabdomyosarcoma [52], neuroblastoma [53] and esophageal cancer [7,11,30]. These observations suggest that SEs can be used to define cancer-type and -subtype identity. Importantly, analysis of SEs-driven TFs can identify cell-type-specific CRC, which will be discussed below.

1.3. Transcriptional Core Regulatory Circuitry (CRC)

Defining epigenomic characteristics including enhancer usage is instrumental to dissecting gene regulatory programs which contribute to activating cellular processes important for cancer biology. SEs participate in transcriptional regulatory network by cooperation with cell-type-specific master TFs and transcriptional co-factors, including chromatin remodelers and modifiers. Many master TFs are autoregulated by binding to their own SE constituents in a 3D genome organization, allowing SEs to be in close contact with the target promoters [11,54–56]. A small group of autoregulated master TFs form an SEs-based CRC, determining cell-type-specific state and cancer biology in malignant cells.

The concept of CRC is matured from the research on pluripotent transcriptional regulatory network of ESCs [6,57–59] and transcriptional dependencies of cancer [16,60]. Initial modeling of CRCs was based on the identification of OCT4, SOX2, and NANOG (OSN) target genes and transcriptional regulation of human ESCs using OSN ChIP-Chip data [6]. It had been hitherto established that OSN functionally regulated genes they trio-occupied (that is, co-occupancy by all three factors). Moreover, OSN bound to their own genes, forming interconnected autoregulatory loops. Based on the similar strategy, CRCs were later constructed in hepatocytes [60] and T-cell acute lymphoblastic leukemia (T-ALL) [16].

These studies reveal the distinguishing features of CRC (Fig. 2): 1) each of CRC TFs is auto-regulated through binding to its own SE; 2) CRC TFs bind to SEs of those of the other core TFs, forming an interconnected auto-regulatory loop; 3) CRC TFs co-occupy their target genes. Based on these features, two mathematical algorithms - “CRC Mapper” [15] and “Coltron” [12] - have been developed for the identification of CRC TFs and CRC network. CRC network can be reconstructed by the following steps: (a) annotation of SE-associated TFs by mapping SE profiles using ChIP-seq, (b) identification of auto-regulated master TFs by sequence-motif scanning [61], (c) designation of CRC TFs by scoring TF connectivity at SE regions, and (d) validation of interconnected regulation among CRC TFs by biological verification including genetic manipulation and functional assays.

The major differences of these two methods are the demarcation of SE regions for sequence-motif analysis and the definition of auto-regulated master TFs. Specifically, the “Coltron” program scans TF binding motifs within the nucleosome-free regions (NFRs) rather than the entire SE domains as used by the “CRC Mapper”. Furthermore, “Coltron” requires auto-regulated TFs to have at least

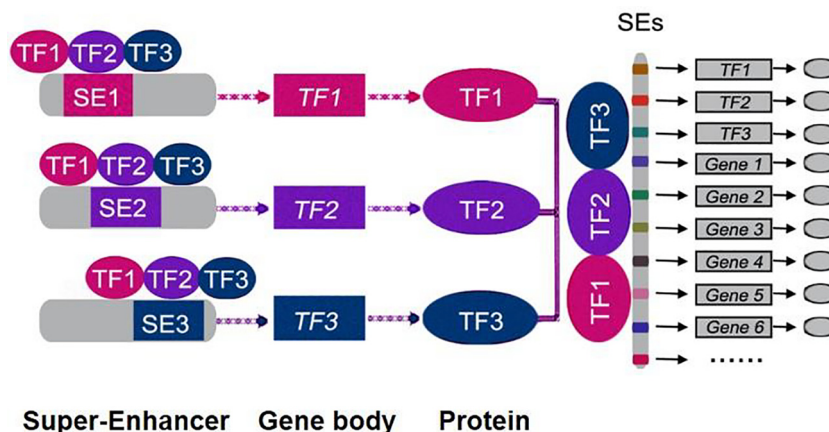


Fig. 2. Feed-forward core transcriptional circuitry (CRC) model. CRCs are assembled to be interconnected loops by several auto-regulated TFs and TF-associated SEs to coordinate gene expression programs that determine cell identities. SE: Super-Enhancer; TF: Transcription Factor.

1 TF binding motif across NFRs inside SE, while “CRC Mapper” asks for 3 TF binding motifs across its whole SE region. As a result, the “Coltron” program requires additional chromatin accessibility datasets such as DHSs or ATAC-seq for the annotation of NFRs. An online database, CRCs-dbCoRC (<http://dbcorc.cam-su.org>), which contains CRC models from 238 human and murine samples on the basis of “CRC Mapper” algorithm, has been established [62].

These approaches are valuable given that they have been utilized to identify many cell-type specific CRCs which are successfully validated. Nevertheless, several caveats exist which need to be considered: 1) Motif scanning has limited predictive value because only a minor portion of motif-enriched regions are actually bound by corresponding TFs; 2) The results are biased toward the well-studied TFs which may lead to false negative results. Indeed, not all TFs have established motifs and well-characterized TFs have more accurate motif sequences compared with those poorly studied; 3) Often members from the same TF family recognize almost the identical motif sequence, such as GATA and AP1 family; 4) SE spans large genomic segments (often over 50 kb), but TF occupancy only occurs at nucleosome-free regions (NFRs), which are typically shorter than 300 bp [63].

Considering these weaknesses and caveats, we recommend to: 1) incorporate either DHS or ATAC-seq data to identify NFRs within SE regions for motif enrichment analysis (which is required by Coltron); 2) take into account the expression levels or regulatory activity (can be inferred by methods such as VIPER) [64] of TFs; 3) use available TF ChIP-seq data for verification. Since enhancers do not always interact with their nearest promoters and can have long-range interaction with distant genes, ChIP-seq, chromosome conformation assays (3C, 4C-seq, ChIA-PET or Hi-C) and functional analysis are necessary to validate the result.

1.4. Cancer-Type and -Subtype Specificity of CRC

Based on the above mathematical algorithms, advanced epigenomic assays and biological verification experiments, cell-type-specific CRC models have been established in both normal and malignant cells [5–16]. Interestingly, the same TFs may participate in different CRC models in a cell-type-specific manner. For example, KLF5 has been shown as a CRC TF with elevated activity in both esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), the two major subtypes of esophageal cancers. However, the cooperativity and co-regulation between master TFs within the CRC network are cancer-subtype specific. Specifically, KLF5 cooperates with ELF3, GATA6 and EHF in the EAC subtype CRC program [7], while in ESCC, KLF5 forms a CRC with TP63 and SOX2

[11]. Similarly, SOX2-containing CRCs have been well-characterized in ESCs [5,6] (together with NANOG and OCT4), ESCC [11] (with TP63 and KLF5) and Glioblastoma [65] (with KLF4, EGR1 and NOTCH1). These observations suggest that the same TFs may be involved in different CRC complex by collaboration and cooperation with different master TFs to regulate cell-type-specific transcriptional programs.

In addition, inter-tumor heterogeneity of CRC programs has been observed in cancers. In medulloblastomas, subgroup-specific CRCs have been identified [12,66]: WNT (LEF1, MAF, RUNX2, EMX2), SHH (OTX1, BARHL2, MAFF, GLI2), Group 3 (OTX2, NRL, CRX) and Group 4 (LMX1A, LHX2, EOMES), providing valuable molecular insights to the clinically-recognizable heterogeneous identities of this brain cancer. Such inter-tumor heterogeneity of CRC has also been observed in other types of cancers, including AML [49–51] and two subtypes of esophageal cancers (ESCC and EAC) [7,11]. These studies demonstrate the subtype-specificity of SEs and CRC complex, highlighting their vital contribution to cell-type-specific transcriptional programs.

In ESCC samples, Jiang et al. observed another putative CRC TFs including MYC, JUNB and FOSL1 in addition to the TP63/KLF5/SOX2 CRC complex [11]. MYC, JUNB and FOSL1 bound to their own SEs, and cross-bound to the SE regions of the other factors. They also exhibited strong positive correlation at their expression level. It is thus possible that two different CRC models exist in ESCC considering its known tumor heterogeneity [67–70]. Indeed, comprehensive molecular analyses have clustered ESCC tumors into two major and one minor molecular subtypes [67–69]. Therefore, the two CRC models in ESCC might represent two different subtypes of ESCC, wherein one is controlled by TP63/SOX2/KLF5 circuit and the other by MYC/JUNB/FOSL1 circuit. These findings suggest that CRC mapping could be used to establish tumor heterogeneity, providing novel insights into subtype-specific cancer biology.

1.5. SEs Mediate Cell-Type-Specific CRC

The classical and important characteristic of CRC is the assembly of interconnected loops including core TFs and TF-associated SEs. However, the mechanisms by which the specificity of CRC is determined remain incompletely understood in most of tumor types. As mentioned above, SEs play critical roles for establishing cell-type-specific identity and function. In a given cell type, specific enhancer modules are selectively utilized to regulate gene expression programs for defining cell identity [53,55,71–73]. For example, in neuron cells, distinct subsets of *c-fos* enhancers are employed to promote *c-fos* transcription in response to different stimuli [55].

Similarly, distinct blood enhancer cluster modules lead to cell-type-specific regulation of MYC expression during hierarchical development of normal and leukemic hematopoietic stem cell [71]. Using 3C and 4C-seq, coupled with CRISPR/Cas9-mediated genomic editing, Jiang et al. uncovered functional long-range loops between distal enhancers and the *TP63* promoter, demonstrated the essential role of three SE constituents of *TP63* in regulating the expression of CRC TFs and tumorigenesis of ESCC [11], and highlighted SEs as an integral component within the ESCC regulatory network.

TP63 is a lineage-specific master TF, which not only promotes tumorigenesis in squamous cells, but also regulates epidermal commitment in normal development. In ESCC, SEs of *TP63* are co-occupied by CRC TFs (TP63, SOX2 and KLF5) and three SE constituents are required for the transcription of TP63 as well as ESCC progression. Intriguingly, of the three functional enhancers, two (e7 and e8) are evolutionary conserved, which are bound by Cebpα, Cebpβ and Pou3f1 and repress TP63 expression in layer-specific keratinocytes [74–75]. These reports suggest that constituent elements of SEs may function in distinct combinations and modules in different cell types; the same SE constituents may also play diverse roles through recruiting different TFs. Not surprisingly, constituent elements of SEs show cell-type-specific genomic localization. For instance, SEs of the *Myc* oncogene are located 1.7 Mb downstream from the *Myc* promoter in AML cells [29,40,71], while a 538 kb region upstream of its promoter is required for the regulation of *Myc* in intestine and prostate cancers [76].

2. Summary and outlook

Gene transcription is a complex and highly coordinated process, requiring concerted activity of various transcriptional activators and repressors on specific DNA regulatory elements [77,78]. CRC consists of SEs and SE-associated master TFs as well as other regulators. In this multi-unit complex, master TFs coordinate transcriptional regulation and chromatin accessibility through protein-protein interactions and chromosomal interactions between SEs and target promoters. To date, CRC models and associated master TFs have been defined in multiple cancer types and subgroups [12–13,15,37,51]. Importantly, pharmacological disruption of CRC selectively suppresses both the expression of tumor-dependent CRC TFs and tumor growth in neuroblastoma [10], chronic lymphocytic leukemia [13], squamous-like pancreatic cancer [79], esophageal cancer [7,11] and liposarcoma.

In future studies, it is important to delineate the mechanistic basis of the *de novo* acquisition of cancer-specific SEs and CRCs in different cancer types. In addition, interacting proteins with functional eRNAs and pharmacological targets of CRC components should be interrogated. Whether and how SEs and CRCs contribute to tumor metastasis, drug resistance and relapse are unknown and warrant investigations. Due to the specificity of regulatory pattern of CRC, rational therapeutic strategies may need to focus on the interface between proteins and SEs.

CRedit authorship contribution statement

Yuan Jiang: Conceptualization, Investigation. **Yan-Yi Jiang:** Conceptualization, Resources, Visualization, Funding acquisition. **De-Chen Lin:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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