Characterization of a Weak Allele of Zebrafish cloche Mutant

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Abstract

Hematopoiesis is a complicated and dynamic process about which the molecular mechanisms remain poorly understood. Danio rerio (zebrafish) is an excellent vertebrate system for studying hematopoiesis and developmental mechanisms. In the previous study, we isolated and identified a cloche172 (clo172) mutant, a novel allele compared to the original cloche (clo) mutant, through using complementation test and initial mapping. Here, according to whole mount in-situ hybridization, we report that the endothelial cells in clo172 mutant embryos, although initially developed, failed to form the functional vascular system eventually. In addition, further characterization indicates that the clo172 mutant exhibited weaker defects instead of completely lost in primitive erythroid cells and definitive hematopoietic cells compared with the clo15 mutant. In contrast, primitive myeloid cells were totally lost in clo172 mutant. Furthermore, these reappeared definitive myeloid cells were demonstrated to initiate from the remaining hematopoietic stem cells (HSCs) in clo172 mutant, confirmed by the dramatic decrease of lyc in clo172 runx1w84x double mutant. Collectively, the clo172 mutant is a weak allele compared to the clo15 mutant, therefore providing a model for studying the early development of hematopoietic and vascular system, as well as an opportunity to further understand the function of the cloche gene.

Introduction

Hematopoiesis is a complicated and dynamic process, including an early primitive wave and a later definitive wave, which occurs in a number of anatomic locations and produces all types of blood cells throughout the lifetime of an animal [1–3]. In vertebrates, hematopoiesis originates from the ventral mesoderm (VM) and it has been proposed that the hematopoietic and endothelial cells arise from a common progenitor, termed hemangioblast [4–6]. In mice, definitive hematopoiesis is believed to be originated from an intra-embryonic tissue known as the aorta-gonad-mesonephros with the presence of the first hematopoietic stem cell (HSC) [2,7,8]. The HSCs then migrate to the fetal liver, the main hematopoietic organ during fetal life, and finally home to the bone marrow, where they undergo further expansion and differentiation into mature blood cells shortly after birth [2,8]. Despite extensive studies, the molecular mechanisms of hematopoietic development and the genetic programs governing the specification, migration and survival of HSCs in these hematopoietic compartments remain poorly understood.

Danio rerio (zebrafish), a freshwater tropical fish that has features suitable for N-Nitrosourea (ENU) mutagenesis mediated large-scale forward genetic screening, is an excellent vertebrate model for studying developmental mechanisms of the hematopoietic and cardiovascular system [9–12]. Moreover, the hematopoietic program is highly conserved between zebrafish and mammals [13,14], so the study of zebrafish hematopoiesis and vascular system development would also contribute to our understanding of this process in higher organisms.

The formation of vertebrate blood vessel can be subdivided into two distinct processes, vasculogenesis and angiogenesis [15]. In vertebrates, the differentiation of the hemangioblasts from the mesoderm and their subsequent migration to form the main axial vessels, which will then undergo lumen formation and artery-vein differentiation, is named "vasculogenesis" [16]. After vasculogenesis, the following sprouting and growth of new vessels from the pre-existing vessels named angiogenesis [17]. Zebrafish vascular system originates from the formation of bi-potential cells – hemangioblast from the ventral mesoderm (VM) at 6 hpf in gastrulation stage [18]. By 12 hpf these cells migrate to lateral plate mesoderm (LPM) where they differentiate to angioblast [18]. By 16 hpf, these angioblasts in LPM converge in the midline of vascular cord located between dorsal ectoderm and notochord [18]. By 28–30 hpf, the dorsal aorta (DA) and the posterior cardinal vein (PCV) can be discerned and are fully lumenized [19].

Similarly to mammals, zebrafish hematopoiesis also consists of primitive and definitive programs, and generates differentiated...
cells analogous to most of the mature blood lineages found in mammals [20]. Zebrafish primitive erythropoiesis originates from the posterior lateral mesoderm (PLM) as a pair of bilateral stripes at 5-somite stage [11]. These stripes subsequently extend anteriorly and posteriorly, and converge in the midline at 20-somite stage to form the main structure of the intermediate cell mass (ICM) where the erythroid progenitors further proliferate and differentiate, enter blood circulation, and finally mature at around 5–7 days post fertilization (dpf) [21]. Zebrafish primitive myelopoiesis arises from the anterior lateral mesoderm (ALM) at 10-somite stage and produces mainly macrophages and neutrophils [22,23]. Zebrafish definitive hematopoiesis is believed to initiate from the ventral wall of dorsal aorta (VDA), an equivalence of the mouse aorta-gonads-mesonephros (AGM), with the formation of HSCs from the hemogenic endothelial cells at 26–30 hours postfertilization (hpf) [24,25]. By 2 dpf, these HSCs in the ventral wall of DA migrate to the posterior blood island (PBI) (also referred to as caudal hematopoietic tissue, CHT) [26] located between caudal artery and caudal vein, and finally home to kidney, the adult hematopoietic organ in zebrafish, by 5 dpf [27,28].

In the previous study [29], we have isolated a differentiated myeloid lineage marker lysozyme C (lyc) -deficient mutant by whole-mount in situ hybridization (WISH), cloche172 (clo172), which was further confirmed as a new cloche mutant allele by complementation tests and positional cloning experiments [29]. The zebrafish cloche (clo) mutant, named for its bell-shaped heart, carries a spontaneous mutation characterized by severe deficiency in endothelial and blood cells, as well as the endocardium[30]. clo acts upstream of the genes important for hematopoietic and vascular development in zebrafish, including stem cell leukemia hematopoietic transcription factor (scl), the homeobox gene (hhex), GATA binding protein 1 (gata1), fetal liver kinase 1 (flk1), friend leukemia integration 1 (fli1) and ETS1-related protein (etsrp) [13,31–34]. In addition, the defects in clo during vascular development are cell-autonomous, whereas those in blood cell development are both cell- and non-cell-autonomous [35]. All of these studies indicate that clo affects hematopoietic and endothelial cell development at a very early stage, which may be at the level of the hemangioblast. Unfortunately, the exact gene responsible for the clo mutant remains unknown owing to its telomeric location on chromosome 13 [31,32]. A recent study showed that lysocardio-lipin acyltransferase (lycat) mRNA partially rescue the blood lineage in clo mutants [32]. Although lycat is the earliest known player in the generation of both endothelial and hematopoietic lineages [32], direct evidence suggesting that lycat is responsible for the clo hematopoietic phenotype is still lacking.

Here we report that, by further characterization of both vascular and hematopoietic development in clo172, the clo172 mutant is a weak allele of the clo mutant, showing varying degrees of developmental defects in endothelial and hematopoietic cells, especially in the myeloid lineage cells. The vascular cells in clo172 mutant were initially developed but failed to form the final functional vessels. For hematopoietic development, the primitive erythroid cells were less affected compared to the primitive myeloid cells which were totally lost in clo172. However, the definitive hematopoiesis including erythroid and myeloid cells reappeared to some extent in clo172 mutant. Combined with the examination of hematopoietic stem cell marker c-myb expression, we speculate that definitive hematopoietic cells are derived from the remaining HSCs in clo172. Consistent with our hypothesis, the c-myb-positive myeloid cells were greatly reduced in 3 dpf clo172 mutants compared with that in the clo172 mutant, confirming the definitive origin of those hematopoietic cells. In summary, the clo172 mutant, carrying a novel allele compared to the clo5 mutant, presents a weak endothelial and hematopoietic phenotype. The clo172 mutant not only provides a model for studying early development of primitive and definitive hematopoietic cells, but also an opportunity to further understand the function of the clo gene.

Results

The vascular development in clo172 mutant embryos is partially defective

The zebrafish clo mutant is notable as its defects in both endothelial and blood cells at a very early stage. Therefore, we firstly explored the development of vascular system in clo172. Flk1, a member of the vascular endothelial growth factor (VEGF), was utilized to perform the whole mount in situ hybridization to characterize the development of endothelial cells and their progenitors in clo172 mutant. The flk1 cells were found in the trunk region in clo172 up to 19.5 hpf, which called the DA and PCV later stage, although its expression level was lower than that in siblings (Figure 1A, B). In 1 dpf wild type embryos, the endothelial cells were present in the head, DA, PCV and intersegmental vessels (ISV) (Figure 1D). In clo172 mutant embryos, the anterior of head, DA and PCV were clearly expressing flk1 but broken in line (Figure 1E), which indicated that endothelial cells were formed in situ but unable to connect into a tube. In 2 dpf wild type embryos, the dorsal longitudinal anastomotic vessels (DLAV) also expressed flk1 (Figure 1G). The flk1+ cells in 2 dpf clo172 mutant embryos were faintly stained in ISV and DLAV (Figure 1H), which suggested that vascular system development was affected in the clo172 mutant embryos. In clo5 mutant embryos, however, the expression of flk1 was completely absent except in lower trunk and tail region (Figure 1C, I). The temporal and spatial expression pattern of flk1 in clo172 mutant embryos suggests that vasculogenesis, the de novo formation of vessel, is initiated but the subsequent connection is blocked, which may be related to the lumen formation and later remodeling process.

clo172 mutant shows severe defects in primitive myelopoiesis but partial defects in erythropoiesis

To uncover the difference of hematopoietic process between clo5 and clo172 mutant, we first characterized the development of primitive blood cells in clo172 mutant at different developmental stages. The temporal and spatial expression of the erythroid lineage marker bcl in the posterior lateral mesoderm (PLM) as two stripes at the 14 hpf stage (Figure 2A) and then merged to form the intermediate cell mass (ICM) at 1 dpf (Figure 2D), as well as in VDA region at 36 hpf (Figure 2G). Expression of bcl in clo172 mutant embryos was partially decreased at corresponding stages (Figure 2B, E, F) but significantly lost in clo5 mutant embryos (Figure 2C, F, I). The different bcl expression between the mutants indicates that primitive erythropoiesis in clo172 mutant is partially defective but not completely disrupted as that in clo5 mutant.

The differentiated myeloid lineage markers l-plastin (Figure 3) and lyc (Figure S2) were utilized to detect the primitive myelopoiesis in clo172 mutant embryos [36,37]. In the sibling embryos, up to 19.5 hpf, the primitive myeloid cells reside in the anterior cephalic mesoderm (Figure 3A, S2A). As embryos develop, myeloid cells migrate onto yolk sac syncytial layer and colonize the posterior part of tail at 1 dpf (Figure 3D, S2D), and further appear in VDA region at 36 hpf (Figure 3G). Largely in accordance with clo5 mutants (Figure 3C, F, I, Figure S2C, F, I), the l-plastin+ and lyc+ myeloid cells were undetectable in clo172 mutants at 19.5 hpf and 1 dpf (Figure 3B, E, Figure S2B, E) compared to that in the same stages of siblings, whereas rare cells were observed in the tail region at 36 hpf clo172 mutants (Figure 3H).
Collectively, the development of erythroid cells was not so severely affected as that of myeloid cells in the clo172 mutant embryos during primitive hematopoiesis, in contrast to the seriously defective development of both lineages in the clo5 mutant.

Myeloid lineage is partially recovered in clo172 mutants during Definitive hematopoiesis

Our previous study [29] has showed that the red blood cells were present in VDA region in clo172 mutant at 2 dpf (Figure S1), we therefore performed the WISH experiment to further examine the development of definitive hematopoiesis in clo172 mutant. Since all the hematopoietic cells are arisen from a common ancestor known as hematopoietic stem cells (HSCs), we firstly investigated the development of HSCs in clo172 mutant. In zebrafish definitive hematopoiesis, c-myb was characterized as the stem cell marker with its presence in the VDA at around 36 hpf[24,38]. In sibling embryos, c-myb+ cells are arranged in a line along the VDA at 36 hpf (Figure 4A) and assembled in tail region named as PBI at 3 dpf (Figure 4D). WISH indicated that the expression of HSC marker c-myb decreased greatly at 36 hpf and slightly at 3 dpf stage in clo172 mutant embryos (Figure 4B, E). In contrast, the expression of cmyb was completely lost in clo5 mutant embryos (Figure 4C, F). These data indicated that HSCs are present but reduced in clo172 mutant when compared to wild type embryos.
Figure 3. Primitive Myelopoiesis in clo^{172} and clo^{s5} mutant embryos. (A–I) Myeloid lineage marker l-plastin expression at 19.5 hpf (A: the left arrow show anterior cephalic mesoderm, B–C), 1 dpf stage (D: the left arrow show anterior cephalic mesoderm, E–F), and 36 hpf stage (G: the left arrow show anterior cephalic mesoderm H–I) in sibling, clo^{172} and clo^{s5} mutant embryos. Embryos are shown with anterior to the left and dorsal up. Inserts are high magnification (20 x) of the corresponding boxed region (the right arrow show tail region).

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Figure 4. Definitive hematopoiesis in clo^{172} and clo^{s5} mutant embryos. (A–C) Stem cell marker c-myb expression at 36 hpf and 3 dpf stage in wild type (A: arrow show VDA region; D: arrow show PBI region), clo^{172} (B, E) and clo^{s5} mutant (C, F) embryos. (G–I) WISH of b{e1} expression at 4 dpf stage in sibling (G: arrow show PBI region), clo^{172} (H) and clo mutant (I) embryos. (J–L) WISH to detect l-plastin expression at 4 dpf stage in sibling (J: arrow show thymus), clo^{172} (K) and clo^{s5} mutant (L) embryos. (M–O)WISH of T lymphocyte marker rag1 expression at 4 dpf stage in sibling (M: arrow show thymus), clo^{172} (N) and clo^{s5} mutant (O) embryos. Embryos are shown with anterior to the left and dorsal up. Inserts are high magnification (20 x) of the corresponding boxed regions. VDA: ventral wall of dorsa aorta; PBI: posterior blood island.

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Therefore, we speculate that HSCs were partially formed in clo\textsuperscript{172} mutant embryos.

To examine our speculation, we further characterized clo\textsuperscript{172} mutants to determine the development of definitive hematopoietic cells that derived from the HSCs. As shown in figure 4 and figure S3, in wild type embryos, the expression of βε\textsuperscript{1+} (red blood cell marker), l-plastin\textsuperscript{+} and lyc\textsuperscript{+} cells (myeloid cell marker) were normally localized in PBI region at 3 and 4 dpf stage (Figure 4G, J, Figure S3 A, D).

No obvious differences in βε\textsuperscript{1+} expression were observed between clo\textsuperscript{172} mutants and siblings, except that red blood cells located in the VDA region (Figure 4H) at 4 dpf, which may be due to the lack of blood circulation in the clo\textsuperscript{172} mutants. In contrast, βε\textsuperscript{1+} cells were nearly undetectable in the clo\textsuperscript{s5} mutant (Figure 4I). On the other hand, the examination of the expression of myeloid lineage markers l-plastin and lyc indicated that myeloid cells were decreased but ectopically expressed in a gradient manner in VDA in clo\textsuperscript{172} mutants at 3 dpf and 4 dpf (Figure 4K, Figure S3 B, E) compared with wild-type siblings, whereas they were also undetectable in the clo\textsuperscript{s5} mutant at 3 dpf (Figure 4L, Figure S3 C, F). Furthermore, investigation of the T-lymphocyte developmental marker rag1 revealed that it was totally lost in thymus in both clo\textsuperscript{172} and clo\textsuperscript{s5} mutants at 4 and 5 dpf (Figure 4N, O, Figure S3 H, I). These data reveal that definitive erythropoiesis and myelopoiesis, but not lymphocyte, are more or less defective in the clo\textsuperscript{172} mutant embryos. Additionally, the development of the definitive myeloid lineage was obviously different from its primitive counterpart.

Taken together, the results suggest that hematopoietic cells partially develop in the clo\textsuperscript{172} mutant during definitive hematopoiesis, which shows a weak phenotype compared to the clo\textsuperscript{s5} mutant.

Definitive myeloid lineage cells in clo\textsuperscript{172} mutant originated from the remaining HSCs population

Since the HSCs were partially formed in the clo\textsuperscript{172} mutant, we therefore continued to examine whether the presented definitive myeloid cells were differentiated from the remaining HSCs. Thus, we introduced runx1 mutation, the well-known gene required for the definitive hematopoiesis in zebrafish\cite{39,40}, into the clo\textsuperscript{172} mutant background by crossing clo\textsuperscript{172} (++/-) mutant with runx1w84x mutant\cite{41}, and then examined the development of definitive myeloid cells in their offspring. The embryos showing clo\textsuperscript{172} mutant morphology change (no circulation, edema of heart) were firstly selected and fixed at 3 dpf, and applied to WISH to detect lyc. The number of lyc\textsuperscript{+} cells was counted and each fish was genotyped for runx1 mutation. The results showed that lyc\textsuperscript{+} cells were dramatically decreased in PBI region of

![Figure 5. Analysis of the origin of definitive myeloid cells in clo\textsuperscript{172} mutant. (A, B) lyc expression pattern at 3 dpf runx1/clo\textsuperscript{172} double mutant (A). WISH of lyc expression in siblings (A–i), clo\textsuperscript{172} mutant (A–ii), clo\textsuperscript{172} (-/-) runx1 (+/-) wild (A–iii), double homozygous mutant (A–iv). Histogram of lyc\textsuperscript{+} cells number (means) in siblings(B, purple column), clo\textsuperscript{172} mutant (B, green column), clo\textsuperscript{172} (-/-) runx1 (+/-) wild (B, pink column) and double homozygous mutant (B, blue column). There are significant differences between double mutant and other groups (**p<0.05), but no difference between clo\textsuperscript{172} mutant and clo\textsuperscript{172} (-/-) runx1 (+/-) wild group (p>0.05). The corresponding case numbers were shown by n in column. doi:10.1371/journal.pone.0027540.g005](https://www.plosone.org/figure5)
The clo^72 mutant embryos are identified by a lack of blood circulation but no obvious morphological changes (Figure S1B) before 30 hpf. Similar to clo^5 mutants, clo^72 mutants gradually exhibit slight swelling of the heart and morphological changes from 2 dpf onward and thereafter (Figure S1E, F). Although the morphological changes are similar to those in clo^5 mutants, the red blood cells are clearly visible and localized in the VDA region of clo^72 mutants (Figure S1E). These data (detailed description in Materials S1) suggested that the clo^72 mutant was a different allele compared to the clo^5 mutant. Thus, the clo^72 mutant was used for further characterization of vasculogenesis and hematopoiesis, and the results were compared to those from clo^5 mutants to identify differences between these two mutants.

WISH of flk1 in clo^72 mutants revealed that vascular endothelium cells were able to form in situ but unable to connect into tubes. We predict that vascular system development is normally initiated but finally fails to form the functional vascular system, which may be defective in lumen formation and later remodeling process.

Unlike clo^5 mutants, in which the development of all lineages in hematopoiesis is severely defective, the clo^72 mutant shows partial defects in both primitive and definitive erythropoiesis. By contrast, myelopoiesis is more complicated in the clo^72 mutant. An interesting issue raised by this study is that the development of primitive myeloid cells is seriously affected, whereas the definitive myelopoiesis is partially restored in the clo^72 mutant. According to the expression of HSC marker c-myb, we observed dramatically decreased but still retained c-myb^+ cells in the clo^72 mutant during definitive hematopoiesis. Due to the remaining HSCs present in the clo^72 mutant, we predicted that definitive erythropoiesis and reappeared myeloid cells might originate from them. Based on Nancy A. Speck’s report[39], which noted that runx1 was required for the emergence of hematopoietic stem cells (HSCs) from hemogenic endothelium during embryogenesis, we speculated that hemC cells in the 3 dpf clo^72 mutant would greatly decrease in PBI region due to the loss of runx1 gene. To validate our speculation, we crossed the runx1^w84x mutant with clo^72 mutant and examined the expression of hemC in their offspring. The results showed that hemC myeloid cells were dramatically reduced in PBI region at 3 dpf stage in runx1^w84x clo^72 double mutant, therefore confirming their definitive origin through the runx1 dependent manner. In addition, it is notable that the blood cell migration is probably dependent on blood flow [42]. Hence, we speculate that visible red blood cells and reappeared myeloid cells existing in the VDA region in the clo^72 mutant might be caused by the lack of blood circulation.

Our study has provided evidence that the clo^72 mutant is a weak allele compared to the clo^5 mutant with partially developed primitive and definitive hematopoiesis, thus providing a model for studying the early development of hematopoietic and vascular system, as well as an opportunity to further investigate the function of clo gene in genetic pathways.

### Materials and Methods

**Ethics statement**

All experimental protocols and animals used in this research were approved by Ethical Committee of Southern medical University (LS2011–030).

### Zebrafish husbandry

Zebrafish were raised and handled as previously described [43,44]. The cloche mutant used in this study was kindly provided by Dr. Didier Y.R. Stainier’s laboratory (University of California at San Francisco). The runx1^w84x mutant [41] was used to cross with clo^72 (+/-) to generate clo^72 (+/-) runx1^w84x (+/+).

### In vitro synthesis of antisense RNA probes

Antisense RNA probes were prepared by in vitro transcription according to a standard protocol [43]. The following probes were used in the study: digoxigenin (DIG)-labeled antisense β-f1-globin, lyC, l-plastin, rag-1, c-myb, runx1 and flk1.

### Whole-mount in situ hybridization

Embryos at different stages were used to examine hematopoietic-related gene expression by WISH as previously described[43]. Consistent with the Mendelian law of heredity, the ratio of mutant to total embryos was approximately 1/4 (Table 1).

### Genotyping of runx1 gene

The clo^72 (+/-) embryos were picked out from clo^72 (+/-) runx1^w84x (+/-) offspring based on the phenotype of no circulation system.

### Table 1. No. of embryos used in WISH.

| Probe | Stage of embryos | clo^72 mutant (mutant/total) | clo^5 mutant (mutant/total) |
|-------|------------------|-----------------------------|-----------------------------|
| flk1  | 21 s             | 39/156                      | 31/133                      |
| flk1  | 1 dpf            | 28/117                      | 24/102                      |
| flk1  | 2 dpf            | 25/104                      | 23/97                       |
| βf1   | 14 hpf           | 52/219                      | 54/221                      |
| βf1   | 1 dpf            | 37/156                      | 35/149                      |
| βf1   | 36 hpf           | 58/208                      | 54/200                      |
| βf1   | 4 dpf            | 34/132                      | 38/145                      |
| l-plastin | 19.5 hpf      | 23/94                       | 29/123                      |
| l-plastin | 1 dpf            | 39/152                      | 41/163                      |
| l-plastin | 36 hpf          | 53/206                      | 49/197                      |
| l-plastin | 3 dpf            | 41/171                      | 38/154                      |
| l-plastin | 4 dpf            | 37/152                      | 33/149                      |
| c-myb | 36 hpf           | 37/152                      | 34/147                      |
| c-myb | 3 dpf            | 27/116                      | 31/127                      |
| rag-1 | 4 dpf            | 22/93                       | 25/103                      |
| rag1  | 5 dpf            | 48/189                      | 44/181                      |
| lyC   | 19.5 hpf         | 36/159                      | 40/165                      |
| lyC   | 1 dpf            | 43/165                      | 41/162                      |
| lyC   | 3 dpf            | 53/209                      | 49/201                      |

**M**: No. of mutant embryos; **T**: No. of total embryos.

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at 28 hpf. Then these embryos were used to check the level of lyce by WISH. After washing with PBST, the genomic DNA was extracted from each embryo for the PCR amplification using nucI primers followed: 5‘-TGGTGGGCAAACTGCGCATG-3’ and 5‘-TCTTGTGCTTGACACTGAGC-3’. Subsequently, the genotype of sibling versus mutants was easily distinguished by the different size of the PCR product after digestion by the Hae II enzyme.

Supporting Information

Figure S1 Dynamic morphological changes of clo172 and clo s5 mutant. [A–F] Lateral view of morphological changes 30 hpf stage of sibling (A), clo172 (B), clo s5 mutant (C) and 2 dpf stage of sibling (D), clo172 (E: arrow show edema heart and red blood cell in VDA region), clo s5 mutant (F: arrow show edema heart).

Figure S2 Expression of lyce during primitive hematopoiesis in clo172 and clo s5 mutant embryos. (A–F) Whole-mount in situ hybridization of lyce expression at 19.5 hpf (A: arrow show anterior cephalic mesoderm, B–C), 1 dpf stage (D: the left arrow show anterior cephalic mesoderm, E–F) in sibling, clo172 mutant and clo s5 mutant embryos. Embryos are shown with anterior to the left and dorsal up. Inserts are high magnification (20×) of the corresponding boxed regions.

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Materials S1 Supporting materials.

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Author Contributions

Conceived and designed the experiments: WZ. Performed the experiments: NM, ZH. Analyzed the data: NM, ZH, W. Liao. Contributed reagents/materials/analysis tools: W. Liu, KW, LZ, HR, SL, XX. Wrote the paper: NM.
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