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Response to Matters Arising: Intercellular genetic tracing of cardiac endothelium in the developing heart

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Studies suggest that the heart tube in *Drosophila* functions as a hematopoietic organ and that the zebrafish endocardial layer gives rise to hematopoietic-like cells. However, the presence of hemogenic activity in mammalian endocardium is controversial, as two studies by Liu et al. failed to find Runx1$^+$ cells and the hemogenic activity of the endocardium using genetic lineage-tracing methods. Here, we discuss the limitation of genetic lineage tracing using the Cre-Rosa26-based system and other methods and how the technical limitations could have resulted in different interpretations. This Matters Arising Response addresses the Liu et al.[11] Matters Arising paper, published concurrently in *Developmental Cell*.

Previous reports suggest that the heart tube serves as a hemogenic organ in *Drosophila*.[1,2] The dorsal vessel (heart equivalent) of *Drosophila* is an established site of hematopoiesis, and *Drosophila* blood cells are predominantly plasmatocytes (macrophages/monocytes) and tinman (Nkx2-5) dependent. In zebrafish, an electron microscopic study found hematopoietic-like cells in the endocardial layer.[3] The authors speculate that these hematopoietic-like cells in the heart originate from the endocardium, as the cells with an intermediate phenotype were also found in the endocardium. This cardio-hematopoietic mechanism may be conserved in mammals.[4–9] Data by us and others show that a subset of endocardial cells express markers for hemogenic endothelial cells and progenitors including Runx1 and CD41 in the atrioventricular and outflow cushion endocardium at around E9.5–E10.5 and give rise to hematopoietic cells and macrophages in a Nkx2-5-dependent manner.[5,6,9]

However, controversy exists as to the hemogenic activity of the mammalian endocardium. While we and others have reported that a subset of endocardial cells expresses hematopoietic regulators including Runx1,[5,6] the Liu et al. Matters Arising paper in this issue is unable to find Runx1$^+$ cells in the endocardium using the Nfatc1-ires-Cre line and the contact-based lineage tracing using the gTCCC system.[10,11] Lack of labeling this critical population led to the conclusion that there is no hemogenic activity in the mammalian endocardium. Data both

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DECLARATION OF INTERESTS
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supporting and rejecting the idea of hemogenic endocardium use similar sets of experimental systems. However, each of these methods has technical limitations. Electron microscopic imaging and immunostaining only represent a snapshot of dynamic behavior of embryonic cells at one time point. Hematopoietic colony assay requires optimization of cytokines and OP9 feeder and is subject to the risk of contamination of the hematopoietic progenitors from circulation.

Cre-Rosa26-based genetic lineage tracing is no exception in that it has limitations. First, there is no Cre line that is perfectly specific for one cell lineage. A combination of two or several lineage indicators would not guarantee specificity. Second, the Cre-Rosa26 reporter system may not be as sensitive as scientists long thought. For example, Isl1 was originally considered to be a lineage-specific marker for the second heart field (SHF) based on Cre-Rosa26-based lineage tracing,[12] but an alkaline phosphatase reporter cassette knocked into Gata4 locus clearly demonstrated that Isl1 also labels the first heart field (FHF) lineage.[13] Another example includes the Mesp1-Cre line. Although Mesp1 is expressed broadly in precardiac mesoderm, the genetic lineage-tracing experiments often show patchy labeling at later stages. The sensitivity of Cre-Rosa26 system is determined by two factors: (1) Strength and duration of the Cre driver expression: Cre driven by genes with low copy number and transient expression may exhibit a lower recombination efficiency. This is particularly problematic for certain transcription factors including Isl1 and Mesp1. (2) The intensity of the reporter: Fluorescent reporters seem to be less sensitive than reporters with an enzymatic reaction such as alkaline phosphatase and β-galactosidase, which involve an amplification process. Third, at the practical level, we occasionally experience ectopic recombination and TAM-independent recombination.

In the previous (2022) report by Liu et al. the Runx1$^+$ endocardial cells were not captured by genetic lineage tracing using Rosa26-LSL-tdTomato line crossed with Nfatc1-ires-Cre, Npr3-CreER, and Cdh5-2A-Cre lines.[10] This can be because of the relatively low expression level of *Nfatc1*, *Npr3, and Cdh5* in the hemogenic endocardial cells; the suppression of the genes encoded after ires; and/or the sensitivity of the tdT reporters used. In their gTCCC lineage-tracing study,[11] the receiver signals are based on the expression levels of Nfatc1 or Cdh5, which are both relatively low in the putative hemogenic endocardial cells. The reporter line is Rosa26-LSL-tdTomato, which is not highly sensitive. In addition, as represented by their images, the Tnnt2-based sender line does not effectively label the neighbor cells in the atrioventricular and outflow cushions, where we identified endocardial cells expressing CD41, runx1, Isl1, and Nkx2-5.[5] The other Cdh5-based sender line is not expected to efficiently label the putative hemogenic endocardial cells, as Cdh5 level is low in this population. In either case, the lack of labeling *Runx1*$^+$ population in Nfatc1-ires-Cre and possibly gTCCC lines resulted in the failure of identifying lineage-labeled hematopoietic cells and macrophages.[10,11] Thus, this controversy is possibly attributed to the sensitivity of the genetic lineage-tracing system.

Identifying the origin of cells with a mobile nature is often difficult. For example, the hematopoietic transition of the endothelial cells in aorta-gonad-mesonephros (AGM) region has been a prevailing theory since its proposal in 1981.[14] However, the smoking gun was not provided for 30 years, until the emergence of hematopoietic cells from endothelia was

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captured by live imaging observation of animals and cell culture.[15–18] The live imaging of the beating embryonic heart is challenging, but further development of technologies will one day give us a definitive answer for the concept of hemogenic endocardium.

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