Establishment of a highly efficient hematopoietic differentiation model from human embryonic stem cells for functional screening

PANG SuLei¹, WU QingQing¹, TIAN Sha², SU Pei¹, BAI Yang¹, GAO Jie¹, YANG YiQing¹,
LIU Xin¹, ZHU ZhengMao², XU YuanFu¹ & ZHOU JiaXi^{1*}

¹State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China;

²College of Life Sciences, Nankai University, Tianjin 300071, China

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Human embryonic stem cells (hESCs) have been successfully differentiated into hematopoietic progenitor cells with colony formation capacity and further into various kinds of blood cells including erythrocytes, megakaryocytes, neutrophils, nature killer cells and T lymphocytes [1–7]. Nevertheless, the differentiation efficiency is extremely low. The differentiation usually involves complex steps such as embryonic body formation or co-culture with stromal cells, causing inconsistencies and difficulties in dissection of its molecular mechanisms [8,9]. Therefore, it is essential to establish a well-defined hematopoietic differentiation model that is independent of serum and stromal cells and much easier to manipulate genes for functional screening, thus the underlying molecular mechanism of hematopoietic differentiation of hESCs could be dissected more easily.

In this study, to avoid inconsistencies caused by feeder cells and serum replacement in the culture medium, we maintained H1 hESCs on matrigel-coated plates in a chemically defined medium, mTeSR. For the initiation of differentiation, hESCs were re-plated as single cells in custom mTeSR medium with various concentrations of growth factors (50 ng mL⁻¹ BMP-4, 50 ng mL⁻¹ Activin A, 40 ng mL⁻¹ VEGF, 50 ng mL⁻¹ bFGF). At 24 h of differentiation, mRNA levels of mesoderm marker *brachyury* (*T*) and *MIXL1* were drastically up-regulated and maintained at a

high level for a further 72 h (Figure 1A). As shown by immunostaining, almost 100% of the cells were positive for brachyury (*T*) (Figure 1A). Generation of uniform mesodermal precursors facilitates further hematopoietic differentiation. Indeed, CD34⁺CD31⁺ cells, which have been recognized as hematopoietic precursors derived from hESCs [10], emerged on day 4 of differentiation and constituted ~25% of the cell population on day 6 (Figure 1B). The terminal differentiation potential of the derived hematopoietic progenitors was verified by generation of various types of colonies, including CFU-E (erythroid), CFU-M (macrophage), and CFU-GM (macrophage/granulocyte) (Figure 1B). Therefore, hematopoietic progenitors with terminal differentiation potential were successfully derived as monolayer independent of stromal cells and sera.

As mentioned above, the major advantage of differentiation as monolayer is greater accessibility for gene manipulation to facilitate mechanistic studies. To explore whether highly efficient RNA interference can be achieved in our monolayer differentiation model and further affect hematopoietic differentiation, we designed five shRNAs targeting the *brachyury* (*T*) gene and tested their interference efficiencies. Strikingly, two shRNAs, T1093 and T2350, interfered with *brachyury* (*T*) expression quite efficiently at both mRNA and protein levels (>90%) (Figure 1C, data not shown). Furthermore, interference of *brachyury* (*T*) expression severely impaired further hematopoietic differentiation.

*Corresponding author (email: zhoujx@ihcams.ac.cn)

Figure 1 Efficient hematopoietic differentiation and RNA interference of human embryonic stem cells. **A**, mRNA levels of *brachyury* (*T*) and *MIXL1* measured by real-time PCR and immunostaining of *brachyury* (*T*) after hematopoietic differentiation of hESCs. Scale bar, 59 μ m. **B**, Hematopoietic differentiation of H1 hESCs. The percentage of CD34⁺CD31⁺ cells was measured by flow cytometry after 6 d of induction. Hematopoietic precursors were applied to colony formation assays. CFU-E, erythroid; CFU-M, macrophage; CFU-GM, macrophage/granulocyte. Scale bar, 200 μ m. **C**, Western blotting of *brachyury* (*T*) protein after RNA interference with shRNAs. **D**, Percentage of CD34⁺CD31⁺ cells measured by flow cytometry after RNA interference of *brachyury* (*T*) (* P <0.05). All experiments were independently repeated three times.

Only ~5% CD34⁺CD31⁺ cells were generated, which was much lower than that in the control group (~25%, P <0.05) (Figure 1D). After *brachyury* (*T*) interference, CD34⁺ cells were further sorted and applied to colony formation assays. Interestingly, we found that no colonies formed after two weeks of culture in medium containing methylcellulose (data not shown). These results suggested that hematopoietic precursors generated with *brachyury* (*T*) interference lost their terminal differentiation potential. Taken together, our results show that this stepwise hematopoietic differentiation model in a monolayer can be used for highly efficient RNA interference. Recently, using small molecules, Wang et al. reported a three-step strategy to induce the differentiation of hESCs into hematopoietic progenitors. They demonstrated that retinoic acid and transforming growth factor- β signaling pathways are important regulators in hematopoietic differentiation of hESCs [11,12]. These studies revealed important signaling pathways during hESC differentiation, but there are obvious limitations because only a limited number of chemical inhibitors are available to manipulate signaling pathways, which hinders functional screening at the single

gene level. In our monolayer system, as indicated by efficient RNA interference of the *brachyury* (*T*) gene, shRNA targeting of genes can be used for functional screening to identify important regulators of hematopoietic differentiation of hESCs. Our system should be able to facilitate functional screening and mechanistic dissection of human hematopoietic development.

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