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Transcriptional regulation of Hhex in hematopoiesis and hematopoietic stem cell ontogeny

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ABSTRACT

Hematopoietic stem cells (HSCs) emerge during development via an endothelial-to-hematopoietic transition from hemogenic endothelium of the dorsal aorta (DA). Using in situ hybridization and analysis of a knock-in RedStar reporter, we show that the transcriptional regulator Hhex is expressed in endothelium of the dorsal aorta (DA) and in clusters of putative HSCs as they are specified during murine development. We exploited this observation, using the Hhex locus to define *cis* regulatory elements, enhancers and interacting transcription factors that are both necessary and sufficient to support gene expression in the emerging HSC. We identify an evolutionarily conserved non-coding region (ECR) in the Hhex locus with the capacity to bind the hematopoietic-affiliated transcriptional regulators Gata2, SCL, Fli1, Pu.1 and Ets1/2. This region is sufficient to drive the expression of a transgenic GFP reporter in the DA endothelium and intra-aortic hematopoietic clusters. GFP-positive AGM cells co-expressed HSC-associated markers c-Kit, CD34, VE-Cadherin, and CD45, and were capable of multipotential differentiation and long term engraftment when transplanted into myelo-ablated recipients. The Hhex ECR was also sufficient to drive expression at additional blood sites including the yolk sac blood islands, fetal liver, vitelline and umbilical arteries and the adult bone marrow, suggesting a common mechanism for Hhex regulation throughout ontogenesis of the blood system. To explore the physiological requirement for the Hhex ECR region during hematoendothelial development, we deleted the ECR element from the endogenous locus in the context of a targeted Hhex-RedStar reporter allele. Results indicate a specific requirement for the ECR in blood-associated Hhex expression during development and further demonstrate a requirement for this region in the adult HSC compartment. Taken together, our results identified the ECR region as an enhancer both necessary and sufficient for gene expression in HSC development and homeostasis. The Hhex ECR thus appears to be a core node for the convergence of the transcription factor network that governs the emergence of HSCs.

1. Introduction

Stem cells are essential for homeostasis of adult organs and also have roles in development, disease, and regeneration. They have the remarkable potential to develop into a specific array of differentiated cell types and maintain this property through successive cell divisions. Hematopoietic stem cells (HSCs) are amongst the best characterized adult stem cells (Bryder et al., 2006). However, despite years of work, a detailed understanding of the molecular regulation of HSC ontogeny is still lacking. Such an insight would guide the in vitro generation of HSCs from pluripotent stem cells (PSCs), affording novel sources of HSCs for transplantation. In addition, it may inform efforts to regulate

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HSC behaviour in the context of stem cell expansion and provide mechanistic insights into HSC dysregulation in malignancy.

During mouse embryonic development, HSCs first emerge in clusters of hematopoietic cells which appear to bud from the floor of the dorsal aorta, from a specialized subset of endothelium, the hemogenic endothelium. Recently, live imaging visualized this process in in vitro and in vivo models of hematopoiesis, capturing the formation of blood cells from endothelium in real time (Bertrand et al., 2010; Boisset et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009). This process appears to occur without obvious cell division (Eilken et al., 2009) through a so-called endothelial-hematopoietic transition (Kissa and Herbomel, 2010). Prior to the generation of HSCs, different types of uni-, bi-, and multi-lineage progenitor cells are generated in the yolk sac, placenta, vitelline and umbilical arteries and heart (Dzierzak and Speck, 2008; Medvinsky et al., 2011; Nakano et al., 2013). These include, among others, the primitive erythrocytes that are formed in the E7.5 blood islands of the yolk sac and the erythro-myeloid progenitors (EMPs) that emerge from the hemogenic endothelium of the yolk sac one day later (Palis et al., 1999; Silver and Palis, 1997). EMPs populate the fetal liver and are critical to the survival of the embryo (Chen et al., 2011). Definitive HSCs are present in the fetal liver from E11 onwards (Sanchez et al., 1996) and move to the fetal bone marrow (BM) shortly before birth, where they maintain the lifelong production of all blood lineages.

The homeobox transcription factor Hhex is expressed in a range of multipotent hematopoietic progenitor cells and cell lines, as well as in endothelial cells of the embryonic vasculature. Its expression is transient and is downregulated during differentiation (Bedford et al., 1993; Crompton et al., 1992; Manfioletti et al., 1995; Thomas et al., 1998), suggesting a role for this gene in the early stages of hematopoietic cell differentiation (Kubo et al., 2005). Indeed, analysis of microarray data from the Immunological Genome Project revealed Hhex expression in multiple hematopoietic lineages, with highest levels in hematopoietic stem and progenitor cells, and developing B cells (Goodings et al., 2015). Moreover, Hhex appears to be required for maturation and proliferation of definitive hematopoietic progenitors from ES-derived hemangioblasts, with Hhex-null embryoid bodies showing a decreased ability to form definitive hematopoietic colonies in vitro (Guo et al., 2003; Kubo et al., 2005) at least in part because Hhex-null hematopoietic progenitors accumulate in the G2 phase of the cell cycle (Paz et al., 2010). Conditional deletion experiments revealed a requirement for Hhex in adult hematopoietic stem and progenitor cell differentiation with defects particularly apparent in the lymphoid compartment of Hhex deficient animals (Goodings et al., 2015; Jackson et al., 2015). Both studies revealed that loss of Hhex increased HSC proliferation while the functional ability of HSCs to contribute to multi-lineage engraftment in transplant was compromised, with one study showing failure of myeloid and lymphoid engraftment (Jackson et al., 2015) and the other an impact on lymphoid engraftment only (Goodings et al., 2015).

Hhex is also recognized as a major regulator of many other aspects of early embryonic development, as an essential regulator of both anterior-posterior axis formation and endoderm development (Zamparini, 2006). While a transcriptional role for Hhex in hematopoiesis has not been demonstrated, its expression pattern suggests that its induction may be a node in the gene regulatory network governing the generation of hematopoietic cells. Using a transgenic approach, Rodriguez et al. showed that the dynamic patterns of Hhex expression during early murine development were regulated by a number of distinct *cis*-acting elements, and suggested that a blood island enhancer was present within an 8 kb region of the Hhex locus. This region also drove reporter gene expression in the AGM (Rodriguez et al., 2001). Indeed, using a computational approach, Donaldson et al. identified a region within Hhex intron 1 that had similarities to the *Scl* enhancer (Donaldson et al., 2005). This element could be used to drive reporter gene expression in fetal liver cells and could bind hematoendothelial transcription factors in vitro. However, the extent to which this or other regions in the Hhex gene are required for expression in authentic HSCs during homeostasis or during their specification in ontogeny remains unknown.

Given the evidence that Hhex is a critical regulator of hematopoietic cell development, maturation and proliferation, we sought to dissect the mechanisms that regulate dynamic Hhex expression in embryonic and hematopoietic differentiation. We found that Hhex was expressed in the endothelium and functional HSCs, and describe an evolutionarily conserved regulatory element that binds HSC-associated transcription factors Fli1, Pu.1, Scl, Ets1/2, Gata1, and Gata2 and was both necessary and sufficient for hematopoietic expression of Hhex. This element thus provides both an experimental tool for forced gene expression in these compartments and a route into further understanding and dissection of their regulatory networks.

2. Methods

2.1. Embryo and adult tissue staining

Embryos were obtained from timed matings as previously described (Rodriguez et al., 2001). Embryos were embedded for either wax or cryo-sectioning. In situ hybridization on wax sections was carried out as previously described (Brickman et al., 2000). Hhex probe was generated from the Hhex ENE1 plasmid, linearized and then transcribed with T3 polymerase. For immunostaining on sections, embryos were fixed in 4% PFA, washed with PBS, incubated in 15% sucrose, embedded in OCT compound and snap-frozen on dry-ice/ethanol. Sections were stained with the nuclear dye, TO-PRO-3 (Molecular Probes) and incubated with CD31 (clone MEC 13.3 BD Pharmingen), CD34 (clone RAM34, BD Pharmingen) and anti-rat Cy3 (Jackson laboratories) primary antibodies. Images were taken on SPE inverted Leica confocal microscope.

For analysis of AGM tissues, embryos were harvested at 10.5–11 dpc. The AGMs were dissected, incubated for 45 min at 37 °C in 0.125% collagenase (Type I; Sigma) in PBS/10%FCS, and made into a single cell suspension. Cells were washed in PBS/10%FCS and spun twice at 1000 rpm. The pellet was resuspended in PBS/10%FCS. For analysis of 14 dpc fetal livers, tissues were harvested passed through a 100 μm cell strainer (BD Pharmingen). For analysis of bone marrow, femur, tibia and hip bones were dissected from adult mice (9–16 weeks old). Bone marrow was flushed from bones using a 25-gauge needle and IMDM media.

Hhex genomic sequences for mouse, rat and human were obtained from NCBI and chicken, zebrafish and fugu were obtained from Ensembl, and the frog sequence was obtained by probing a BAC library. Genomic sequences were aligned to the mouse sequence using the Vista alignment tool (www-gsd.lbl.gov/vista/).

2.2. Transgenic model generation

The Hhex-GFP plasmid generated by Rodriguez et al. (2001) was digested to derived different transgenic constructs containing the eGFP gene cloned in frame into the exon 1 of the Hhex gene, and which spans 4.2 kb upstream and 3.8 kb downstream of the endogenous promoter.

Mouse transient transgenic embryos and transgenic founder lines were generated via pronuclear injection into (CBAxC57BL/6)/F2 zygotes following standard procedures. Transient transgenic embryos and F0 founder males were genotyped by PCR on genomic DNA. Transgene expression in embryos was assessed by fluorescent microscopy. Transgenic lines were established and maintained on a mixed (CBAxC57BL/6) background.