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Epiblast-specific loss of HCF-1 leads to failure in anterior-posterior axis specification

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ABSTRACT

Mammalian Host-Cell Factor 1 (HCF-1), a transcriptional co-regulator, plays important roles during the cell-division cycle in cell culture, embryogenesis as well as adult tissue. In mice, HCF-1 is encoded by the X-chromosome-linked *Hcfc1* gene. Induced *Hcfc1*$^{cKO/+}$ heterozygosity with a conditional knockout (cKO) allele in the epiblast of female embryos leads to a mixture of HCF-1-positive and -deficient cells owing to random X-chromosome inactivation. These embryos survive owing to the replacement of all HCF-1-deficient cells by HCF-1-positive cells during E5.5 to E8.5 of development. In contrast, complete epiblast-specific loss of HCF-1 in male embryos, *Hcfc1*$^{epiKO/Y}$, leads to embryonic lethality. Here, we characterize this lethality. We show that male epiblast-specific loss of *Hcfc1* leads to a developmental arrest at E6.5 with a rapid progressive cell-cycle exit and an associated failure of anterior visceral endoderm migration and primitive streak formation. Subsequently, gastrulation does not take place. We note that the pattern of *Hcfc1*$^{epiKO/Y}$ lethality displays many similarities to loss of β-catenin function. These results reveal essential new roles for HCF-1 in early embryonic cell proliferation and development.

1. Introduction

In animals, early embryonic development is associated with rapid rounds of cell division, which allow the multicellular embryo to acquire cell numbers sufficient to support cell differentiation and development. These rapid rounds of cell division often short circuit cell-cycle regulators particularly of the G1 phase. Consequently, many G1-phase cell-cycle regulators such as transcriptional activators (e.g., E2Fs), repressors (Retinoblastoma protein (pRb) pocket-protein family), and repressors of repressors (e.g., cyclin–CDK complexes) are not required for early developmental events before embryonic day (E) 8.5, including gastrulation (reviewed in Ciemerych and Sicinski (2005)).

Here, we study a broadly active transcriptional co-regulator called HCF-1 encoded by the X-chromosome-linked *Hcfc1* gene in mice (Frattini et al., 1996; Kristie, 1997). HCF-1, in human a 2035 amino acid protein first identified as a host-cell factor for herpes simplex virus infection (reviewed by Wysocka and Herr (2003)), is required for the proliferation of cells in culture (Goto et al., 1997; Julien and Herr, 2003), at least in part, by its ability to associate with both DNA sequence-specific (e.g., E2F1 and E2F4, THAP11/Ronin, Myc) and chromatin-modifying (e.g., MLL and Set1 histone H3 lysine 4 methyltransferase, Sin3 histone deacetylase and BAP1 deubiquitinase) transcriptional regulators (reviewed by Zargar and Tyagi (2012); see also Thomas et al. (2015)). In culture, HCF-1 is required for both passage from G1 to S phase (Goto et al., 1997) and proper passage through M phase (Reilly and Herr, 2002); promotion of G1-to-S phase passage is linked to the ability of HCF-1 to associate with E2F proteins (Knez et al., 2006; Tyagi et al., 2007; Tyagi and Herr, 2009) and THAP11 (Parker et al., 2014).

We have recently described a conditional knock-out (cKO) mouse allele called *Hcfc1*^{lox}, where the presence of Cre recombinase induces deletion of two essential exons leading to the predicted synthesis of a small inactive truncated 66 amino acid HCF-1 peptide (Minocha et al., 2016). *Hcfc1* expression is ubiquitous in embryonic and extraembryonic tissues (Minocha et al., 2016). Because the *Hcfc1* gene resides on the X chromosome, female offspring carry two *Hcfc1* alleles of which one or the other is randomly inactivated at around E4.5–E5.5 (Clerc and Avner, 2011), whereas male offspring only possess one allele, which remains active throughout development. Epiblast-specific inactivation of the *Hcfc1*^{lox} allele (generating an *Hcfc1*^{epiKO} allele) by E5.5 does not reduce the viability of heterozygous females but is embryonic lethal in male embryos (Minocha et al., 2016). In the surviving

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heterozygous female embryos, HCF-1-deficient cells are progressively and by E8.5 entirely replaced by HCF-1-positive cells carrying the deleted *Hcfc1*^{epiKO} allele on the inactive X chromosome (Minocha et al., 2016).

The progressive loss of HCF-1-deficient cells in an environment in which half the cells remain positive for HCF-1 could be owing to cell competition if HCF-1-deficient cells cannot replicate as efficiently as their wild-type neighbors (Baillon and Basler, 2014). Such a cell-competition effect has been observed in the mouse epiblast as a result of variable levels of Myc oncoprotein (Claveria et al., 2013). Alternatively, HCF-1-deficient cells may simply fail to replicate. These two possibilities can be distinguished by examining the *Hcfc1*$^{epiKO/Y}$ male embryos where no potentially competing embryonic HCF-1-positive cells are present. If heterozygous *Hcfc1*$^{epiKO/+}$ female embryos eliminate HCF-1-deficient cells by cell competition, the absence of HCF-1-positive competing cells should rescue epiblast cell replication in *Hcfc1*$^{epiKO/Y}$ males at least transiently.

Here, by analyzing *Hcfc1*$^{epiKO/Y}$ male embryos, we describe the specific requirement for HCF-1 in early mouse embryonic development. Generation of the epiblast-specific *Hcfc1*^{epiKO} allele around E5.5 rapidly halts cell-proliferation, leading to developmental arrest by E6.5 prior to gastrulation. Thus, unlike the many aforementioned G1-phase cell-cycle regulators, which are not essential until after gastrulation, HCF-1 function is required for early embryonic development. Indeed, *Hcfc1*$^{epiKO/Y}$ embryonic cells exit the cell cycle earlier than in heterozygous *Hcfc1*$^{epiKO/+}$ female embryos, suggesting that loss of HCF-1-deficient cells in *Hcfc1*$^{epiKO/+}$ heterozygotes is not due to competition. Rather, in the *Hcfc1*$^{epiKO/+}$ heterozygotes, HCF-1-positive cells appear to support the proliferation of their HCF-1-deficient neighbors.

2. Materials and methods

2.1. Mice

All experimental studies have been performed in compliance with the EU and national legislation rules, as advised by the Lemanique Animal Facility Network (Resal), concerning ethical considerations of transportation, housing, strain maintenance, breeding and experimental use of animals.

Homozygous mice bearing the *Hcfc1* conditional (lox) allele are referred as *Hcfc1*$^{lox/lox}$ in this study (Minocha et al., 2016). The *Hcfc1*^{lox} allele contains two loxP sites, one in intron 1 and another in intron 3 that undergo recombination in the presence of Cre recombinase. This removes exon 2 and 3 to generate the conditional knockout (cKO) allele encoding a highly truncated 66 amino acids long HCF-1 protein.

Other strains used include wild-type *C57BL/6* mice and *C57BL/6* mice carrying the *Sox2Cre*^{tg} transgene (Hayashi et al., 2002).

Females and littermate males were housed four to five per cage at 23 °C, with a 12:12h light–dark cycle and ad libitum access to water and food. The day of finding the vaginal plug was assumed to be 0.5 days post-coitum.

2.2. DNA isolation and genotyping

For genotyping, genomic DNA was isolated from mouse ear tags for postnatal mice or entire conceptus for whole embryos as previously described (Truett et al., 2000). For paraffin-embedded embryo sections, DNA for genotyping was extracted by (i) preferential scraping of the epiblast region of 3–4 sections with a surgical blade, (ii) transferring the scraped sections into an eppendorf tube, and (iii) deparaffinizing and xylene removal as described (Minocha et al., 2016). Subsequent DNA extraction was done as described (Truett et al., 2000). Samples were used for PCR amplification with specific primer sets using the KAPA2G Fast HotStart Genotyping PCR Mix (cat no. KK5621). The annealing was done at 62 °C for 15 s with an extension at 72 °C for 10 s.

Primers for genotyping are listed below.

For HCF-1: p1 (5'-GGAGGAACATGAGCTTTAGG-3'), p2 (5'-CAATAGGCGAGTACCATCACAC-3'), and p3 (5'-GGGAAAGTAGACCCACTCTG-3') (Minocha et al., 2016).

For Cre: Sense (5'-AGGTGTAGAGAAGGCACTTAGC-3') and Antisense (5'-CTAATCGCCATCTTCCAGCAGG-3') (Le and Sauer, 2000).

For mouse Y chromosome: Sry-1 (5'-AACAACTGGGCTTTGCACATTG-3') and Sry-2 (5'-GTTTATCAGGGTTTCTCTCTAGC-3') (Steele-Perkins et al., 2005).

2.3. BrdU incorporation

To label embryonic cells during S phase, pregnant mice were injected intraperitoneally 5-bromo-2'-deoxyuridine (BrdU; BD Biosciences, cat. # 550891) to a final concentration of 50 mg/kg body weight, sacrificed 24h post-injection, and BrdU incorporation revealed by immunofluorescence staining (see below).

2.4. Tissue histology and immunohistochemistry

Intact E5.5 to E8.5 embryos were paraffin-embedded and sectioned within their decidua along a saggital axis to generate 4 μm thick sections using a microtome (MICROM HM325). The paraffin-embedded sections were prepared for hematoxylin and eosin (HE) staining, and immunohistochemical detection of proteins.

Paraffin-embedded sections were (i) deparaffinized in xylene, (ii) rehydrated through graded alcohol washes, (iii) rinsed twice with PBS, (iv) antigen revealed by heating in a 750 W microwave oven until boiling (approximately 10 min) in citrate buffer (10 mM, pH 6.0), (v) allowed to slowly cool down at 4 °C, (vi) washed twice with PBS, (vii) blocked for 30 min with 2% normal goat serum (NGS) (Sigma-Aldrich, cat. #G9023) in PBS at room temperature (RT), (viii) incubated with specific primary antibody diluted in 2% NGS overnight at 4 °C, (ix) washed thrice with PBS, (x) incubated with secondary antibody for 30 min in the dark at RT, (xi) washed thrice with PBS, (xii) counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, CAS # 28,718-90-3), (xiii) washed twice with PBS, (xiv) embedded with Mowiol mounting medium (Sigma-Aldrich, CAS # 9002-89-5), and (xv) analyzed using an AxioImager M1 microscope with AxioCam MRm monochrome and AxioCam MRc color cameras (Carl Zeiss AG, Oberkochen, Germany). Images were processed using AxioVision 4.8.2 software (Carl Zeiss AG, Oberkochen, Germany).

Primary antibodies used were: rabbit anti-HCF-1 (1:1000, H12 (Wilson et al., 1993)), rat anti-Ki67 (1:60, eBioscience cat. # 41-5698), mouse anti-HNF4α (1:100, R&D Systems cat. # PP-H1415-00), rabbit anti-Histone H3 phospho Ser10 (1:100, Abcam cat. # ab5176), rat anti-BrdU (1:250, AbD Serotec cat. # OBT0030), and mouse β-catenin (1:50, BD Biosciences, cat. # 610153).

Secondary antibodies used were: Goat anti-rabbit Alexa 488 (1:400, Molecular Probes cat. # A11034), goat anti-mouse Alexa 568 (1:500, Molecular Probes cat. # A11019), goat anti-rabbit Alexa 568 (1:1000, Molecular Probes cat. # A21069), goat anti-mouse Alexa 488 (1:400, Molecular Probes cat. # A11029), and donkey anti-mouse Alexa 594 (1:500, Molecular Probes cat. # A11005).

2.5. TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed on paraffin-embedded embryo sections with the *in situ* cell death detection kit (Roche Applied Science, product # 11684795910), according to the