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# STELLA collaborates in distinct mesendodermal cell subpopulations at the fetal-placental interface in the mouse gastrula

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ABSTRACT

The allantois-derived umbilical component of the chorio-allantoic placenta shuttles fetal blood to and from the chorion, thereby ensuring fetal-maternal exchange. The progenitor populations that establish and supply the fetal-umbilical interface lie, in part, within the base of the allantois, where the germ line is claimed to segregate from the soma. Results of recent studies in the mouse have reported that STELLA (DPPA-3, PGC7) co-localizes with PRDM1 (BLIMP1), the bimolecular signature of putative primordial germ cells (PGCs) throughout the fetal-placental interface. Thus, if PGCs form extragonadally within the posterior region of the mammal, they cannot be distinguished from the soma on the basis of these proteins. We used immunohistochemistry, immunofluorescence, and confocal microscopy of the mouse gastrula to co-localize STELLA with a variety of gene products, including pluripotency factor OCT-3/4, mesendoderm-associated T and MIXl1, mesendoderm- and endoderm-associated FOXa2 and hematopoietic factor *Runx1*. While a subpopulation of cells localizing OCT-3/4 was always found independently of STELLA, STELLA always co-localized with OCT-3/4. Despite previous reports that T is involved in specification of the germ line, co-localization of STELLA and T was detected only in a small subset of cells in the base of the allantois. Slightly later in the hindgut lip, STELLA +/(OCT-3/4+) co-localized with FOXa2, as well as with RUNX1, indicative of definitive endoderm and hemangioblasts, respectively. STELLA was never found with MIXl1. On the basis of these and previous results, we conclude that STELLA identifies at least five distinct cell subpopulations within the allantois and hindgut, where they may be involved in mesendodermal differentiation and hematopoiesis at the posterior embryonic-extraembryonic interface. These data provide a new point of departure for understanding STELLA's potential roles in building the fetal-placental connection.

## 1. Introduction

The allantois of placental mammals will become the umbilical cord, ensuring establishment of a continuous fetal-placental vasculature intimately associated with the maternal circulation. At one end, the allantois is connected to the embryo, where its vasculature becomes confluent with that of the fetus and yolk sac. At the other, the allantois fuses with the chorion, forming the chorio-allantoic labyrinth within the placenta and site of fetal-maternal exchange. While the source of the cells that build the fetal-umbilical connection, including the umbilical vasculature, hindgut endoderm, and canonically segregated primordial germ cells (PGCs), has long been thought to be located within the posterior embryonic primitive streak (Beddington, 1982, 1981; Tam and Beddington, 1987), the latter's contribution of descendant cells to the embryonic-extraembryonic interface is limited (Downs et al., 2009; Kinder et al., 1999; Mikedis and Downs, 2012; Tam and Beddington, 1987). Rather, as demonstrated in recent experiments (Downs, 2009; Downs et al., 2009; Inman and Downs, 2006a; Mikedis and Downs, 2012), the streak's posterior end extends beyond the embryo and into the allantois, where it collaborates with the embryonic posterior primitive streak to provide distinct and overlapping descendant cell types to posterior tissues (Mikedis and Downs, 2012). This putative extraembryonic component of the primitive streak (XPS) is initially identifiable by juxtaposition of morphologically compact cells adjacent to posterior visceral endoderm (PVE), and robust localization of Brachyury (T; Downs et al., 2009). By headfold stages (~E7.75–8.0),

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the XPS expands, becoming the Allantoic Core Domain (ACD; Downs et al., 2009) that is essential for allantoic elongation and contributes to both the allantois itself (Downs et al., 2009) as well as to tissues encompassed by the fetal-placental interface (Mikedis and Downs, 2012).

Consistent with building the embryonic-extraembryonic interface, the ACD exhibits a variety of proteins involved in pluripotency (Downs, 2008; Mikedis and Downs, 2009, 2012), including those thought to signify the germ line (Chiquoine, 1954; Ginsburg et al., 1990; Lawson and Hage, 1994; reviewed in Leitch and Smith (2013)). Initially identified qualitatively on the basis of elevated alkaline phosphatase (AP) enzyme activity (Chiquoine, 1954), PGC progenitors are thought to enter the posterior primitive streak from the epiblast, congregate within the proximal end of the allantois, and there become segregated from the soma (Lawson and Hage, 1994). From the allantois, the PGCs then migrate as a segregated population into the gut as it forms (Lawson and Hage, 1994), and onto the genital ridges, populating the developing gonadal parenchyma (Chiquoine, 1954; reviewed in Mikedis and Downs (2014)). While clonal fate mapping, based on AP activity, has suggested that the site of PGC segregation from the soma lies within the base of the allantois (Lawson and Hage, 1994), rigorous evidence to show continuity via clonal fate mapping from the allantois to the gonads, or even to the hindgut, is absent from the literature (reviewed in Mikedis and Downs (2014)).

For more than six decades, cells within the PGC trajectory have been characterized largely by gene expression and protein localization. AP-positive putative PGCs were found to express several germline-specific “marker” gene products, one of which, STELLA (Saitou et al., 2002), is a demethylation-protective factor (Nakamura et al., 2007) required for pre-implantation development (Bortvin et al., 2004; Payer et al., 2003). However, its abrogation does not yield infertile progeny (Bortvin et al., 2004; Payer et al., 2003), and its role in this population remains unknown.

While the relationship between the PGCs and the allantois has generally been unappreciated, results of recent studies have demonstrated that STELLA protein localizes throughout the posterior region (Mikedis and Downs, 2012) and therefore appears to lack specificity in identifying a segregated germline. OCT-3/4 (Pou5f1), a POU domain transcription factor, has also been claimed to be specific to the germline, as its expression becomes restricted to the area of greatest tissue-nonspecific AP (TNAP) activity by embryonic day 8 in the base of the allantois (Scholer et al., 1990). Oct-3/4 also regulates *Stella* expression in maturing oocytes (Levasseur et al., 2008; Zuccotti et al., 2009). Like STELLA, however, OCT-3/4 localizes to myriad developing tissues of the mouse conceptus (Downs, 2008), including the primitive streak, where it maintains cell proliferation (DeVeale et al., 2013; Downs, 2008). Also, OCT-3/4, like STELLA, is associated with a variety of stem cell populations in mouse development (Garagna, 2009; Scholer, 1991) and pluripotency in embryonic stem cells in vitro (Sterneckert et al., 2012).

While STELLA+ descendants of ACD-derived cells colonize the hindgut, a component tissue of the PGC trajectory, ACD-derived STELLA is also found broadly throughout the fetal-umbilical junction and outside of the hindgut (Mikedis and Downs, 2012, 2013; reviewed in Mikedis and Downs (2014)). Whether the STELLA-positive cells of the ACD represent a pure segregated population, or whether they are subpopulations of cells that collectively build posterior tissues, including the PGCs, is obscure. For example, we have recently demonstrated that STELLA and PRDM1 (BLIMP1), a transcriptional repressor thought to regulate differentiation of progenitor cell populations, co-localize both within and outside of the canonical PGC trajectory (Mikedis and Downs, 2017). However, both STELLA and PRDM1 were also found independently of each other throughout the posterior region, including the allantois and hindgut, again calling into question the accuracy of expecting these proteins to specifically identify segregated putative PGCs.

These results suggest that a variety of molecularly distinct STELLA subpopulations may be present at the fetal-umbilical interface. To begin to identify these, we undertook systematic analysis by immunofluorescence (IF) and immunohistochemistry (IHC) during the presumed period when PGCs segregate within the allantois, then apparently leave this tissue to colonize the hindgut (~E7.5–E9.5). We paid particular attention to the allantois and hindgut, which are thought to encompass the PGC trajectory (McLaren, 2003) and examined a number of gene products, including T, MIXl1 (Pereira et al., 2011; Tada et al., 2005; Wolfe and Downs, 2014), and FOXa2 (HNF3β), a winged helix/forkhead transcription factor (Ang et al., 1993; Besnard et al., 2004) that can be employed as a specific marker of endoderm (Kubo et al., 2004). For reasons described above, we also co-localized STELLA and OCT-3/4, and we co-localized STELLA and Runx1 which identifies hemangioblasts (North et al., 1999) in the allantois and posterior region (Daane and Downs, 2011; Zeigler et al., 2006), and which has been shown to co-localize with PRDM1 (Mikedis and Downs, 2017).

Results of this study revealed distinct subpopulations of STELLA+ cells within the allantois, associated visceral endoderm, and hindgut that may play roles in mesendodermal differentiation of a variety of progenitor subpopulations at the fetal-umbilical junction.

## 2. Materials and methods

### 2.1. Mouse husbandry, dissections, embryo staging

All animals were treated in accordance with Public Health Service Policy on Humane Care and Use of Laboratory Animals (Public Law 99–158) as enforced by the University of Wisconsin-Madison. F2 conceptuses were obtained by mating inbred hybrids of B6CBAF1/J (The Jackson Laboratory, Bar Harbor, ME; Downs, 2006) or *Runx1-LacZ* heterozygous reporter males (Daane and Downs, 2011; North et al., 1999; Zeigler et al., 2006) with F1 females. Individual estrous females (Champlin et al., 1973) and stud males were placed together just before lights went off (13:00/01:00 or 21:00/09:00 lights off/on); copulation plugs were identified up to 12 h later. Dissection and staging of conceptuses were as described by Downs and Davies (1993).

Staged conceptuses were fixed in 4% paraformaldehyde (PFA) for 2 h at 4 °C and sequentially rinsed at least three times in phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO; Downs, 2008). Specimens were then dehydrated in increasing methanols/PBS through absolute methanol and stored at −20 °C for at least three days. Prior to rehydration for protein localization, specimens were opened in the lateral yolk sac and amnion with the bladed bevel of a 27-gauge insulin needle to ensure reagent penetration.

### 2.2. Antibodies for immunofluorescence (IF) and immunohistochemistry (IHC)

Protein localization was evaluated by IF and IHC. Two primary antibodies raised in different species against STELLA were needed for co-localization: **anti-STELLA AF2566** (goat polyclonal, raised against *E. coli*-derived recombinant full-length mouse STELLA/Dppa3; R & D Systems, Minneapolis, MN) used at 1:70 dilution (2.9 μg/mL) for IF (Mikedis and Downs, 2012); and **anti-STELLA sc-67249** (rabbit polyclonal, raised against full-length mouse STELLA; Santa Cruz Biotechnology, Santa Cruz, CA) used at 1:25-1:20 dilution (8–10 μg/mL) for IF. Other primary antibodies included: **anti-OCT-3/4** (goat polyclonal, raised against human N 1–134; sc-8268, Santa Cruz) used at 1:30 dilution (6.7 μg/mL) for IF and 1:100 dilution (2 μg/mL) for IHC (Downs, 2008); **anti-T** (goat polyclonal, raised against N-terminal 19 residues of human Brachyury; sc-17743, Santa Cruz) (Inman and Downs, 2006b) used at 1:25 dilution (8 μg/mL) for IF; **anti-MIXl1** (rabbit polyclonal, raised against mouse MIXl1 N-terminal residues 1-134; sc-98665, Santa Cruz) used at