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Apical constriction in distal visceral endoderm cells initiates global, collective cell rearrangement in embryonic visceral endoderm to form anterior visceral endoderm

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ABSTRACT

The behavior of visceral endoderm cells was examined as the anterior visceral endoderm (AVE) formed from the distal visceral endoderm (DVE) using the mouse lines *R26-H2B-EGFP* and *R26-PHA7-EGFP* to visualize cell nuclei and adherens junction, respectively. The analysis using *R26-H2B-EGFP* demonstrated global cell rearrangement that was not specific to the DVE cells in the monolayer embryonic visceral endoderm sheet; each population of the endoderm cells moved collectively in a swirling movement as a whole. Most of the AVE cells at E6.5 were not E5.5 DVE cells but were E5.5 cells that were located caudally behind them, as previously reported (Hoshino et al., 2015; Takaoka et al., 2011). In the rearrangement, the posterior embryonic visceral endoderm cells did not move, as extraembryonic visceral endoderm cells did not, and they constituted a distinct population during the process of anterior-posterior axis formation. The analysis using *R26-PHA7-EGFP* suggested that constriction of the apical surfaces of the cells in prospective anterior portion of the DVE initiated the global cellular movement of the embryonic visceral endoderm to drive AVE formation.

1. Introduction

After implantation into the uterus, the embryonic portion of the mouse embryo consists of two monolayer sheets, the epiblast and the embryonic visceral endoderm (EmVE), and both adopt a cup shape. The epiblast and EmVE sheets are adjacent to the extraembryonic ectoderm (ExEc) and the extraembryonic visceral endoderm (ExVE), respectively. The embryos are radially symmetric along the distal-proximal axis. ExVE cells are cuboidal, while EmVE cells are squamous. Cell thickening occurs in the distal visceral endoderm (DVE) at E5.5, and this is the initial landmark of the anterior-posterior axis formation in mouse embryos (Rivera-Perez et al., 2003; Rossant and Tam, 2009; Thomas et al., 1998). The DVE cells express a set of unique genes (AVE genes) encoding transcription factors, such as *Otx2* and *Hhex*, and signaling antagonists, such as *Dkk1*, *Cerl* and *Lefty1* (Acampora et al., 1995; Belo et al., 1997; Hoshino et al., 2015; Kimura-Yoshida et al., 2005; Rossant and Tam, 2009; Thomas et al., 1998; Yamamoto et al., 2004). The expression of these genes shifts to the anterior visceral endoderm (AVE) by E6.0, and the AVE suppresses primitive streak formation in the adjacent epiblast and induces it to become the anterior ectoderm (Kimura et al., 2000; Kinder et al., 2001; Perea-Gomez et al., 2002; Tam and Steiner, 1999; Yamamoto et al., 2004). The shift of AVE gene expression from DVE to AVE is not due to changes in the cells that express these genes but to the movement of the DVE cells (Thomas et al., 1998). Time-lapse imaging studies using transgenic lines harboring genes encoding fluorescent proteins under the direction

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of an AVE transcriptional regulatory unit suggest that DVE cells move actively to the future anterior (Hoshino et al., 2015; Srinivas et al., 2004; Torres-Padilla et al., 2007). However, only the DVE cells were visualized in these studies; the studies did not provide information about how other visceral endoderm cells respond to or influence DVE cell migration. A study addressed this question with a *Gata6* BAC transgenic line that expressed TOMATO conjugated with the NH2-terminal sequence of neuromodulin to localize it to the membrane in visceral endoderm cells (Takaoka et al., 2011). Here, we analyzed the behavior of each visceral endoderm cell using the *R26-H2B-EGFP* mouse line, which ubiquitously expresses EGFP in the nuclei of the mouse embryos (Abe et al., 2011; Kurotaki et al., 2007). One of the advantages of H2B fusions as live imaging reporters is that they facilitate the identification and tracking of single cells, while at the same time, they permit the visualization of an entire population. The analysis demonstrated a global, collective cell rearrangement that took place in the embryonic visceral endoderm (EmVE) to form the AVE. Subsequently, we have examined the cell rearrangement with the *R26-PHA7-EGFP* mouse line, which expresses EGFP at the zonula adherens (ZA). The analysis revealed that constriction of the apical surfaces of the anterior DVE cells was associated with the global cell rearrangement.

2. Experimental procedure

2.1. Mouse lines

The *R26-H2B-EGFP* mice were previously described (Accession No. CDB0238K: http://www2.clst.riken.jp/arg/reporter_mice.html) (Abe et al., 2011; Kurotaki et al., 2007). The *R26-PHA7-EGFP* mice (Accession No. CDB0261K) were generated in this study with the *PLEKHA7-EGFP* fusion gene (Meng et al., 2008) as previously reported (Abe et al., 2011); the details will be provided upon request. *R26-PHA7-EGFP* was genotyped as described (Abe et al., 2011). *Otx2* mutant mice were previously described (Accession No. CDB0010K: http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) (Matsuo et al., 1995). The animals were housed in environmentally controlled rooms, and all the experimental procedures using animals were approved by the Institutional Animal Care and Use Committee of RIKEN Kobe Branch.

2.2. Whole embryo culture and time-lapse imaging

The embryos were harvested and cultured as previously described (Hoshino et al., 2015; Shioi et al., 2011). Briefly, E5.5 embryos were embedded and cultured in collagen gels, which were mixed with Dulbecco's Modified Eagle Medium (DMEM) containing 50% rat serum, 1 mM β-mercaptoethanol, 1 mM sodium pyruvate and 100 μM nonessential amino acids. In these culture conditions, as previously reported, the expression of AVE genes, such as *Cer1*, which exists in the DVE at the start of culture, shifts to AVE, and the expression of posterior genes, such as *Brachyury*, which is absent at the start of the culture, occurs in the posterior epiblast after 24 h (Hoshino et al., 2015). The RT-PCR analysis of the marker genes also indicated that the development in the culture is representative of that in a uterus. Time-lapse imaging was carried out using a Confocal Scanner Box CV1000 (Yokogawa), LCV100-CSU10 or LCV110-CSUW1 (Olympus). Images were taken every 3 or 10 min with a 20-fold objective lens, and 11–22 optical sections along the z-axis were taken in 3- or 5-μm intervals. These time-lapse images were analyzed with MetaMorph (Molecular Devices, LLC.), Imaris software (Bitplane) or ImageJ (Schneider et al., 2012).

2.3. Immunohistology

The embryos were collected in DMEM and fixed in 4% paraformaldehyde. The fixed embryos were washed three times with wash buffer (0.1% Triton X-100 in PBS) and blocked in blocking buffer (5% donkey serum in wash buffer). The embryos were incubated overnight at 4 °C with primary antibodies diluted with the blocking buffer. After three washes, the embryos were incubated overnight with DAPI (nacalai tesque) and the appropriate secondary antibodies conjugated with the fluorescent probes Cy3, Cy5, Dylight649 (Jackson ImmunoResearch) or Alexa Fluor-488 (Molecular Probes). The primary antibodies used were goat anti-GFP (Abcam, ab5450) and rabbit anti-β-CATENIN (SIGMA-ALDRICH, C2206). The samples were imaged with a Nikon TiE-A1RSi confocal laser-scanning microscope.

2.4. Statistical analysis

Differences in the cell cycle time among the cells in each E5.5 population were tested by Welch's *t*-test (Ruxton, 2006) and by q-value (Storey and Tibshirani, 2003). The values $P < 0.05$ and $Q < 0.05$ were considered statistically significant. The statistical analysis was done using R statistical software (version 3.3.3, CRAN).

3. Results and discussion

3.1. R26-H2B-EGFP demonstrates global, collective cell rearrangement

We did not observe cell movements in the visceral endoderm of the E5.25 embryos during a 6 h culture; the movement took place at E5.5, with DVE formation, as previously reported (Hoshino et al., 2015; Takaoka et al., 2011). The tracking of the locations of each visceral endoderm cell nuclei was then conducted using the embryos harvested at E5.5 and cultured for 24 h. Fig. 1 gives an example of the tracking with *R26-H2B-EGFP*. At the start of imaging, the visceral endoderm cell nuclei in each domain were colored differentially in the 3D reconstructed images. The thickened DVE cells were green, the cells caudally adjacent to the DVE cells (cDVE) were yellow, the cells in the prospective anterior visceral endoderm (pAVE) were red, the cells in the prospective proximo-lateral visceral endoderm (pLVE) were light blue, the cells in the prospective disto-lateral visceral endoderm (dLVE) were dark blue, the cells in the prospective posterior visceral endoderm (pPVE) were pink and the ExVE cells were gray. After the embryos were cultured for 24 h, the AVE was the domain extending from the anterior boundary between the embryonic and extraembryonic regions to the distal tip. This was based on our previous data of each AVE gene expression in E6.5 embryos (Hoshino et al., 2015). The number of cells randomly chosen in each visceral endoderm domain at E5.5 for the lineage tracking was 30 in the DVE, 23 in the cDVE, 34 in the pAVE, 15 in the dLVE, 28 in the pLVE, 13 in the pPVE and 50 in the ExVE. These were the numbers from three independent experiments with three embryos.

Most of the E5.5 DVE cells (green) migrated to the future anterior and then to the boundary between the EmVE and ExVE by approximately 5 h, and then, subsequently, moved to the lateral region, and some remained in the most anterior portion of AVE (Figs. 1, 2C). Most of the cells located at the AVE after the 24 h culture were derived from the E5.5 cDVE cells (yellow). These features are consistent with previous live imaging studies (Hoshino