Dynamic Heterogeneity of Brachyury in Mouse Epiblast Stem Cells Mediates Distinct Response to Extrinsic Bone Morphogenetic Protein (BMP) Signaling*§

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Mouse pluripotent cells, such as embryonic stem cells (ESCs) and epiblast stem cells (EpiSCs), provide excellent in vitro systems to study imperative pre- and postimplantation events of in vivo mammalian development. It is known that mouse ESCs are dynamic heterogeneous populations. However, it remains largely unclear whether and how EpiSCs possess heterogeneity and plasticity similar to that of ESCs. Here, we show that EpiSCs are discriminated by the expression of a specific marker T (Brachyury) into two populations. The T-positive (T+) and the T-negative (T−) populations can be interconverted within the same culture condition. In addition, the two populations display distinct responses to bone morphogenetic protein (BMP) signaling and different developmental potentials. The T+ EpiSCs are preferentially differentiated into ectoderm lineages, whereas T− EpiSCs have a biased potential for mesendoderm fate. Mechanistic studies reveal that T+ EpiSCs have an earlier and faster response to BMP4 stimulation than T− EpiSCs. Id1 mediates the commitment of T+ EpiSCs to epidermal lineage during BMP4 treatment. On the other hand, Snail modulates the conversion of T+ EpiSCs to mesendoderm fates with the presence of BMP4. Furthermore, T expression is essential for epithelial-mesenchymal transition during EpiSCs differentiation. Our findings suggest that the dynamic heterogeneity of the T+/T− subpopulation primes EpiSCs toward particular cell lineages, providing important insights into the dynamic development of the early mouse embryo.

The early mouse embryo development is a process that in vivo pluripotent cells gradually commit for different lineages. Inner cell mass (ICM)2 from embryonic day 3.5 (E3.5) blastocyst exhibits pluripotency (1). From E4.5 to E5.5, the implanted embryo grows like an egg cylinder structure with pluripotent cells called the epiblast (2, 3). The epiblast at this stage consists of a single layer of histologically homogeneous pluripotent epiblชม cells (4, 5). At E6.5, with the forming of the primitive streak in the posterior embryo, the embryo starts to undergo lineage commitment (1). Three germ layers, including the ectoderm, mesoderm, and endoderm, are established after gastrulation at E7.5 (6). During the gastrulation, epiblast cells that migrate through the primitive streak develop to the mesendoderm. The migrating epiblast cells first loosen the adhesion and then become mesenchymal cells after the constriction and the extrusion, which is termed the epithelial-mesenchymal transition (EMT) (7, 8). The layer of cells that do not ingress into the primitive streak is referred as ectoderm, which is restricted to the epidermal and the neural lineages (9, 10).

Numerous studies have shown that in vitro pluripotent stem cells can recapitulate the in vivo developmental process (11, 12). Mouse embryonic stem cells (ESCs), established from ICM or early epiblast at E3.5 to E4.5, are pluripotent and can give rise to all of the primary germ layers of the embryo proper as well as the germ cell lineage (11, 13). Epiblast stem cells (EpiSCs), derived from the epiblast of the postimplanted mouse embryos between E5.5 and E7.5 (14–16), are pluripotent populations, which could also be differentiated into three germ layers. To maintain undifferentiated status, EpiSCs are kept in culture medium containing activated bFGF and Activin/Nodal signals, which resembles the environment of the anterior primitive streak cells in mouse embryos (16). Besides EpiSCs that derived from the mouse epiblast, EpiSCs could also be established from the differentiated ESCs (12). The ESC-derived EpiSCs (ESD-EpiSCs) have properties similar to the embryo-derived EpiSCs (12). Upon EpiSC differentiation, bFGF or Activin/Nodal signaling is necessary to program EpiSCs to the mesendoderm (17–19), and bone morphogenetic protein (BMP) signaling is also essential to promote mesendoderm fate commitment (9, 20).

Recently, it is becoming evident that heterogeneity in pluripotent stem cells is common (21–23). ESCs exist in multiple interconvertible states, as revealed by the fluctuated expression of Nanog and several other key pluripotent genes (24, 25). ESCs constantly shuttle between ICM and epiblast-like states, depending on the expression of Stella (26). In Rex1/Oct3/4 knock-in ESCs, the conversion among the ICM, the epiblast, and the early primitive ectoderm was also observed (27). The
Results

The Majority of T+ Cells in EpiSCs Are Pluripotent—In our previous study, we have established ESD-EpiSCs from ESC differentiation process (12). Therefore, we generated T-GFP EpiSCs from T-GFP ESCs, in which an EGFP cassette is knocked into the T locus (31). T-GFP ESCs were trypsinized to the single cell suspension, and then the cells were aggregated as embryonic bodies (EBs). Two days later, EBs were seeded in the chemical defined medium (CDM), containing Activin and bFGF (CDM/AF medium). The T-EpiSC clones were formed after culture in the CDM/AF medium for 6 days (Fig. 1A). Typical pluripotent cell characteristics, such as high nucleus/cytoplasm ratio, were observed in GFP-positive T-EpiSCs (Fig. 1A, a and b). Immunostaining assays showed that signals of the endogenous T (red) and T-GFP (green) were exclusively co-localized (Fig. 1A, c–f), indicating that GFP signal is a faithful indicator of T expression. After 14 passages, the percentage of T-GFP positive (T+) cells was about 32%, determined by FACS (Fig. 1B, a). After nine more passages, the proportion of T+ cells had no significant change (Fig. 1B, b). The similar heterogeneous expression of T was observed in another EpiSC cell line that derived from early mouse embryo (14) (data not shown). The results above indicate that T is expressed in EpiSCs.

To answer whether T+ EpiSCs are pluripotent, we performed double immunostaining assays with a T-specific antibody and an antibody against Oct4, a pluripotent marker. The majority of T+ EpiSCs (red) expressed Oct4 (green), and only a few T+ EpiSCs were Oct4-negative (Fig. 1C). Consistently, the similar expression patterns of T/Nanog were also detected in T-EpiSCs (data not shown). These results indicated that the majority of T-positive EpiSCs was pluripotent.

Next, the expression patterns of Oct4 and T were assessed by in situ hybridization in mouse embryos at E6.5, E7.0, and E7.5, stages that were associated with the initiation and the elongation of primitive streak (2, 32). Oct4 was expressed in the whole epiblast at all stages examined (Fig. 1D, a). Consistent with previous reports (32, 33), T was mainly expressed at the posterior-proximal region of embryo at E6.5, and the posterior of embryo at E7.0 and E7.5, which indicated the primitive streak region (Fig. 1D, b). Interestingly, Oct4 and T are obviously co-expressed in the posterior part of the embryo from E6.5 to E7.5 (Fig. 1E, a and b). Taken together, the results above demonstrated that the majority of T+ EpiSCs are positive for Oct4 both in cell culture in vitro and in early mouse embryo in vivo, indicating that those T+ EpiSCs are pluripotent.

Dynamic Expression of T-GFP in T-EpiSCs—Next, we asked whether the T-GFP-negative and the T-GFP-positive populations in EpiSCs shared the same characteristics. T− cells and T+ cells were separated by FACS sorting, and the purity of the two populations was evaluated immediately, with more than 94% purity each (data not shown). The two cell populations were each cultured and monitored in the CDM/AF medium for 48 h. Both cell populations were attached onto a cell culture dish at 12 h after replating. Interestingly, at 24 h, a few GFP-positive cells emerged from the T− population. Meanwhile, a few T+ cells changed to GFP-negative in the T+ population (Fig. 1F). At 48 h, many T-GFP-positive cells were observed in the culture of the T− population; in comparison, there were many GFP-negative cells in the culture of the T+ population (Fig. 1F). These data indicated that T+ and T− EpiSCs cultured in vitro have the ability to interconvert to each other, which is consistent with a recent report (30). To further demonstrate the heterogeneity property of EpiSCs, we randomly selected 36 T-EpiSCs and 38 ESD-EpiSCs to assess their gene expression profiles at the single cell level by real-time quantitative PCR (qPCR). Single cell qPCR assays showed that in both T-EpiSC and ESD-EpiSC populations, the pluripotent marker gene Oct4 as well as the epiblast marker gene Fgf5 were highly expressed in every cell examined, whereas the expression of T was only detected in about 30% of cells (Fig. 1G). These data support the notion that the heterogeneous expression of T exists in EpiSCs.

To further investigate the heterogeneity of EpiSCs, the T-GFP EpiSCs were double-stained with antibodies recognizing SSEA1, which is an ESC pluripotency marker (29, 34), or GFP for cell sorting. Four groups of cells were separated: 57% SSEA1-positive cells (SSEA1+/T−), 21% SSEA1 and T-GFP double-positive cells (SSEA1+/T+), 9% SSEA1 and T-GFP double-negative cells (SSEA1−/T−), and 12% T-GFP-positive cells (SSEA1−/T+). qPCR assays demonstrated that similar expression...
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**A**
- a: GFP
- c: GFP
- e: GFP
- b: GFP
- d: T
- f: DAPI

**B**
- a: GFP
- b: GFP

**C**
- a: T
- c: T/Oct4
- b: Oct4
- d: DAPI

**D**
- E6.5
- E7.0
- E7.5

**E**
- a: E6.5
- b: T
- E7.0
- E7.5

**F**
- T- Cells
- T+ Cells
- 12h
- 24h
- 48h

**G**
- Oct4
- Fgf5
- T

**H**
- a: Epiblast
- b: Mesendoderm
- c: Ectoderm

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levels of pluripotent marker genes Oct4 and Nanog, as well as the epiblast marker genes Fgf5 and Nodal, were detected in all of these four subpopulations, indicating a comparable level of pluripotency (Fig. 1H, a). The expression of the mesendoderm marker genes, such as T, Sox17, Mix1l, and Foxa2, was activated in the T-positive cells (Fig. 1H, b). On the other hand, the expression of ectoderm lineage-related genes Sox1, Six3, CK19, and CK18, especially the neuroectoderm marker gene Six3 and epidermis marker gene CK19, was increased in the SSEA1 and T-GFP double-negative cells (Fig. 1H, c). These observations suggest that EpiSCs consist of distinct pluripotent populations, with the T-positive cells biased to mesendoderm and SSEA1^- T^- double-negative cells biased to ectoderm lineage.

**T^- and T^+ EpiSCs Show Distinct Developmental Potential**—Next, we asked whether T^- cells and T^+ cells have the same or distinct differentiation potential. The T^- cells and T^+ cells were sorted and differentiated in the CDM plus 10% FBS medium for unbiased differentiation for 2 days. The differentiated cells displayed different morphologies in these two populations. A homogenous cell type with stratified epidermis-like morphology was observed in the culture of T^- EpiSCs (Fig. 2A, a), whereas the cells derived from T^+ EpiSCs generated fibroblast-like morphology, indicating a mesenchymal fate (Fig. 2A, b). In addition, qPCR revealed that the expression of the mesodermal markers, Gsc, Flk1, and Mix1l, and the endodermal markers, Gata4, Gata6, and Sox17, was enhanced in the differentiated T^- EpiSCs (Fig. 2B, a and b). In contrast, the expression of the neuroectodermal markers, Sox1, Pou3f1, and Sox2, was up-regulated in the differentiated T^+ EpiSCs (Fig. 2B, c). Similarly, the expression of the epidermal markers, CK15, Zo-1, and CK19, was promoted in T^- cells but not in T^+ EpiSCs (Fig. 2B, d). Furthermore, the single cell differentiating potential of T^- EpiSCs was assessed by seeding a FACS-sorted single T^- cell or T^+ cell in a 96-well dish, respectively. After differentiation for 2 days in the CDM plus 10% FBS medium, 29 single cells were isolated for single cell qPCR assays. As expected, the expression of the mesodermal markers, Gsc and Mix1l, and endodermal markers, Gata4 and Gata6, was induced in the T^- cells (Fig. 2C, a and b). The expression of the neuroectodermal markers, Sox1 and Pou3f1, and epidermal markers, CK15 and CK19, was promoted in the T^- group (Fig. 2C, c and d). The results above suggest that the T^- cells and T^+ cells possess distinct development potential, with the T^- cells biased to the ectoderm lineage and the T^+ cells for mesendoderm fate.

**BMP Treatment Could Promote the Expression of Mesendodermal Genes in T^+ EpiSCs and Epidermal Genes in T^- Cells**—We further asked whether the interconvertible expression of T^- and T^+ EpiSCs could mediate the distinct capabilities of EpiSCs responding to the extrinsic signals. BMPs belong to the TGFβ superfamily and are multifunctional growth factors. BMP signaling induces the mesoderm development in EpiSCs and promotes epidermal specification in ectoderm (9, 10, 12, 35). Given that the interconversion of T^- and T^+ cells occurred 12 h after FACS sorting (Fig. 1F), FACS-sorted T^- and T^+ cells were treated with BMP4 for 3, 6, and 12 h, respectively. Sixteen single cells were isolated from each population at each time point and subjected to single cell qPCR analysis. The endogenous expression of T was used to confirm the GFP-based sorting efficiency. As expected, the expression of T was barely detected in the T^- population, whereas T was readily expressed in the T^+ population (Fig. 3A). The expression patterns of the pluripotent marker Oct4 and the epiblast marker genes Fgf5 and Nodal were similar in both groups (Fig. 3, A and B). Interestingly, the expression of mesoderm marker genes Mix1l and Gsc, and endoderm marker genes Foxa2 and Gata6 was increased gradually in T^- cells with BMP4 treatment (Fig. 3, C and D). In sharp contrast, mesendoderm marker genes were not changed after BMP4 treatment in the T^- cell group, but epidermis marker genes CK14 and CK18 were significantly up-regulated in T^- cells (Fig. 3F). As a response to BMP signaling, neural marker genes Sox1 and Six3 were barely detectable in both T^- and T^+ cells (Fig. 3F). These data indicate that BMP treatment could promote the expression of mesendoderm lineage genes in T^- cells, while accelerating the expression of epidermal fate genes in T^+ EpiSCs.

**T^- and T^+ EpiSCs Exhibit Distinuishable Responses to Extrinsic BMP Signal**—In BMP signaling transduction, BMP ligands bind to a heterotetrameric receptor complex composed of type I and type II receptors to activate intracellular components (36, 37). Then phosphorylated Smad1, -5, and -8 proteins form a complex with Smad4 and are translocated into the nucleus, where they regulate the expression of downstream genes with other transcription factors (38–40). To investigate whether BMP signal activation is different between T^- and T^+ EpiSCs, FACS-sorted T^- and T^+ cells were treated with BMP4, respectively. The expression of Id1 and Id2 genes, two BMP-targeting genes, was gradually up-regulated after treatment with BMP4 for 3 h independent of T expression, indicating the activation of BMP signaling in both groups (Fig. 4A). A Western blotting assay also showed that the levels of phospho-Smad1/5/8 were enhanced in EpiSCs treated with BMP4 for 3 h (Fig. 4B); meanwhile, the levels of FGFR signal effector phospho-ERK

**FIGURE 1.** T^-positive cells are pluripotent in EpiSCs, and the T-GFP positive/negative cells exhibit different gene expression patterns. A, T^-EpiSCs established from T^-GFP ESCRs were continuously maintained for 20 passages. The cellular morphology (a); GFP expression (b); and immunostaining for GFP (green; c) and endogenous T (red; e) or DAPI (f) are presented. Scale bar, 50 μm. B, the percentages of T^-GFP-positive cells in T^-EpiSCs of different passages were analyzed by FACS. The GFP-positive proportion is 32% at passage 14 and 33.4% at passage 23. C, immunocytochemical assays of T (red) and Oct4 (green) in EpiSCs. The arrowhead indicates the T and Oct4 double-positive cells; the arrow indicates the T-positive cells. Scale bar, 10 μm. D, whole-mount in situ hybridization of Oct4 on T in early mouse embryos (EG.5–E7.5). The arrowhead marks the position plane of the transverse section of the corresponding embryo below. Scale bar, 100 μm. A, anterior; P, posterior. E, whole-mount in situ hybridization of mouse embryonic section from EG.6.5 to E7.5. The RNA probes of Oct4 and T were used. Scale bar, 100 μm. F, GFP-positive and GFP-negative cells in T^-EpiSCs were separated by FACS and then cultured in CDM/AF medium. Cellular morphology and GFP fluorescence were detected after seeding for 12, 24, and 48 h, respectively. Scale bar, 20 μm. G, expression level of Oct4, Fgf5, and T in T^-EpiSCs and ESD^-EpiSCs was determined by single cell qPCR. 36 single T^-EpiSCs and 38 single ESD^-EpiSCs were randomly selected and subjected to single cell qPCR analysis. H, gene expression levels of FACS sorted from four subpopulations, including epiblast marker genes Oct4, Nanog, Fgf5, and Nodal; mesendoderm marker genes T, Sox17, Mix1l, and Foxa2; and ectoderm marker genes Sox1, Six3, CK19, and CK18. Error bars, S.E.
FIGURE 2. T-GFP-positive and -negative EpiSCs show different developmental potential. A, GFP-positive and -negative cells in T-EpiSCs were separated by FACS and then cultured in CDM plus 10% FBS medium for 2 days. The morphology of cells that differentiated from the T− and T+ population was shown. Scale bar, 200 μm. B, expression levels of mesoderm, endoderm, neuroectoderm, and epidermis marker genes in three kinds of cells: T-EpiSCs without any treatment (black bar), the cells differentiated from T− population (white bar), and the cells differentiated from T+ population (green bar). The values represent the mean ± S.D. (error bars). C, single cell qPCR assays using the cells described in A. Shown are gene expression levels in the cells differentiated from the T− population (purple) and the cells differentiated from the T+ population (green). Each dot indicates the expression level of one cell.
FIGURE 3. BMP treatment could up-regulate mesendodermal genes in T-positive EpiSCs and increase epidermal gene expression in T-negative cells. A, the T^- (blue) and T^+ (red) cells in T-EpiSCs were separated by FACS and then cultured in CDM with 10 ng/ml BMP4 for 3, 6, and 12 h, respectively. About 16 individual single cells were randomly collected to conduct the single cell qPCR assay in each time point. The expression level of T^+ and Oct4 was examined. Each dot indicates the expression level of one single cell, and the average expression level is shown. B-F, expression levels of epiblast marker genes Fgf5 and Nodal (B), mesoderm marker genes Mixl1 and Gsc (C), endoderm marker genes Foxa2 and Gata6 (D), epidermis marker genes Ck14 and Ck18 (E), and neural marker genes Sox1 and Six3 (F) were examined by single cell qPCR. Each dot indicates the expression level of one single cell. Error bars, S.E.
FIGURE 4. T-GFP-positive and -negative EpiSCs show different responses to BMP signal. A, the T− (gray) and T+ (black) cells in T-EpiSCs were separated by FACS and then cultured in the CDM medium with 10 ng/ml BMP4 for 3, 6, and 12 h, respectively. Expression levels of BMP signal downstream genes Id1 and Id2, were examined by single cell qPCR. B, Western blotting analysis of phospho-Smad1 (p-Smad1), Smad1, phospho-ERK (p-Erk), ERK, phospho-Smad2 (p-Smad2), Smad2, and β-actin in EpiSCs after BMP4 treatment for 3 h. C, the T− and T+ cells in T-EpiSCs were separated by FACS and then cultured in the CDM medium with 10 ng/ml BMP4 for 15, 30, and 60 min, respectively. Shown is Western blotting analysis of phospho-Smad1, Smad1, GFP, and β-actin in EpiSCs after BMP4 treatment. D, expression levels of BMP ligand genes Bmp2, Bmp4, and Bmp7; BMP receptor genes Bmpr1a, Bmpr1b, and Bmpr2; and BMP effector genes Smad1, Smad4, and Smad7 in three kinds of cells: T-EpiSCs (gray bar), FACS-sorted T− population (white bar), and FACS-sorted T+ population (black bar). The values represent the mean ± S.D. (error bars).
or Nodal signal effector phospho-Smad2 were not altered (Fig. 4B).

To clarify whether BMP signal transduction is different between T− and T+ EpiSCs in a short period of time, the FACSorted T− and T+ cells were treated with BMP4 for 15, 30, 60 min, respectively. GFP immunoblotting data showed that T− and T+ EpiSCs were separated successfully (Fig. 4C). Interestingly, phospho-Smad1/5/8 could be detected in T+ cells treated with BMP4 for 15 min, whereas the Smad1/5/8 activation in T− cells did not occur until 60 min after BMP4 treatment, indicating that T+ EpiSCs have an earlier and faster response to BMP4 stimulation (Fig. 4C). Then we investigated whether the T− and T+ cells express different BMP signaling components. qPCR data showed that the expression of BMP ligand genes, including BMP2, BMP4, and BMP7, and BMP receptor genes, such as Bmp1a, -1b, and -2, as well as BMP effector gene Smad1, in T+ EpiSCs was higher than that in T− EpiSCs (Fig. 4D). Meanwhile, the expression of Smad7 in T+ EpiSCs was lower than in T− EpiSCs, and the expression of Smad4 was comparable between the two populations (Fig. 4D). These data indicate that the heterogeneity of T expression in EpiSCs endows different responses to extrinsic signals, such as BMP signaling.

BMP4 Promotes Mesendoderm Lineage Commitment through Snail in T+ EpiSCs and Accelerates Epidermis Fate Differentiation via Id1 in T− Cells—T− and T+ EpiSC subpopulations exhibit distinguished response to extrinsic BMP signal (Figs. 3 and 4). The Snail gene plays a key role in EMT process (8, 41–43). The Id1 gene, a downstream gene of BMP signaling, is essential for the epidermis differentiation (44, 45). To investigate whether Snail or Id1 is associated with the responses to BMP4 in EpiSCs, Snail or Id1 was knocked down in T-EpiSCs by using a CRISPR-Cas9-mediated gene silencing strategy (46, 47). Two sets of gRNAs (gRNA1 and gRNA2) were used to excise the Id1 or Snail gene, respectively, and the expression of Id1 or Snail could be efficiently reduced with corresponding gRNAs in EpiSCs (Fig. 5A, a). The expression levels of the pluripotency markers Oct4 and Nanog, and the epiblast markers Fgf5 and Nodal were comparable in wild-type T-EpiSCs, iCas9-Id1-1/2 T-EpiSCs, and iCas9-Snail-1/2 T-EpiSCs (Fig. 5A, b). Next, the wild-type T-EpiSCs, iCas9-Id1-1/2 T-EpiSCs, and iCas9-Snail-1/2 T-EpiSCs were sorted by T-GFP-based FACS, and then the cells were replated in the CDM in the absence or the presence of BMP4 for 24 h, respectively (Fig. 5B). Compared with T-EpiSCs and iCas9-Id1-1/2 T-EpiSCs, the expression of the mesoderm markers, Mixl1 and Gsc, and the endoderm markers, Foxa2 and Gata6, was reduced in iCas9-Snail-1/2 T-EpiSCs (Fig. 5B, a and b). On the other hand, the expression of the epidermis markers CK14 and CK18 was decreased in iCas9-Id1-1/2 T-EpiSCs, compared with wild-type T-EpiSCs and iCas9-Snail-1/2 (Fig. 5B, c). The expression of neural markers Sox1 and Six3 was not altered with the deletion of either Id1 or Snail in both the T− and T+ subpopulations of EpiSCs (Fig. 5B, d). These results suggest that BMP4 promotes mesendoderm fate determination via Snail in T+ EpiSCs and accelerates epidermis lineage commitment through Id1 in T− cells.

T Is Essential for EMT—In early mouse embryogenesis, T is required for mesoderm formation during the process of gastrulation, accompanied with cellular movement and constriction initiated by epithelial-mesenchymal transition (48–50). To assess whether this behavior was also observed in the pluripotent T-expressing cells in EpiSCs, we analyzed the T+ cell fate commitment tendency during EpiSC differentiation. T expression was knocked down in EpiSCs by using a lentivirus-mediating strategy. Two independent T-knocked down cell lines (T-KD1 and T-KD2) were established. The mesendodermal differentiation of EpiSCs was achieved by culturing EpiSCs as EBs in CDM plus 10% FBS medium for 4 days. The qPCR analysis showed that the endogenous T expression was reduced to about 30% of the control in two knocked down cell lines at differentiation day 1 and day 2 (Fig. 6A). During the differentiation of the control cells, the expression of epithelial marker gene E-cadherin was decreased gradually. Meanwhile, the expression of mesenchymal marker genes N-cadherin and Fibronectin was up-regulated and reached a peak at day 2 or day 3, respectively (Fig. 6A), indicating that the EMT progress is associated with mesendodermal differentiation. In contrast, the reduction of E-cadherin expression was not as significant as that of the control during the differentiation of T-KD1/2 cells, and the enhancement of N-cadherin and fibronectin expression was significantly repressed (Fig. 6A). The expression of Snail and Zeb2 saw the same change (Fig. 6A). Furthermore, Western blotting assays were performed to assess the expression profiles of EMT-related factors during the differentiation of EpiSCs.

Consistent with the expression profiles of their transcripts shown in Fig. 6A, the expression of T protein was decreased dramatically at differentiation day 1 and day 2 in the T-deficient cells, compared with the control cells (Fig. 6B). The expression of epithelial protein E-cadherin was up-regulated significantly at differentiation day 2 after T depletion, accompanied by the reduced expression of mesenchymal marker genes N-cadherin and Fibronectin at day 2 (Fig. 6B). The expression of EMT-promoting factor Snail and FGF-signaling downstream effector phospho-ERK was also decreased at differentiation day 2 and day 3 in T-deficient cells (Fig. 6B). Meanwhile, the expression of mesoderm marker genes Flk1 and Mixl1 and endoderm marker genes Gata6 and Sox17 was decreased in T-knocked down cells (Fig. 6C). Consistent with the qPCR results, immunofluorescence assays confirmed the reduced percentage of T+ cells, Flk+ mesodermal cells, and Gata6+ endodermal cells of differentiated 3-day EBs from T-KD1/2 EpiSCs (Fig. 6D). The results above indicated that EMT is compromised in T-deficient pluripotent EpiSCs, which leads to the repression of mesendodermal fate commitment during EpiSC differentiation.

Discussion

In recent decades, studies have revealed that self-renewal ESCs are not homogeneous; instead, they contain distinct populations in dynamic equilibrium states. Recent findings have provided new insights on the heterogeneous property of EpiSCs (29, 30, 52). However, whether and how the interchangeability of EpiSCs contributes to diverse differentiation responses remains largely unclear. In this study, we demonstrate that the expression of T oscillates in EpiSCs, and different EpiSC subpopulations show distinct responses to BMP signal. Further-

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more, two EpiSC subsets distinguished by the expression of T exhibit different lineage-biased potential. T⁺ EpiSCs prefer to be differentiated into the mesendodermal fate, whereas T⁻ EpiSCs prefer to be committed to the ectoderm lineage (Fig. 7).

T expression is observed in the extraembryonic ectoderm at E5.5 and is detected at the proximal-posterior epiblast at E6.0, where the prospective primitive streak localizes (32). As embryonic development proceeds, T expression is restricted in the primitive streak during gastrulation from E6.5 to E7.5 (Fig. 1) (48, 53, 54). Interestingly, the co-localization of T and pluripotent marker Oct4 in mouse embryos in vivo (Fig. 1) (48) and in epiblast stem cells in vitro (Fig. 1) implies that those T⁺ cells are pluripotent. Furthermore, the characteristics of EpiSCs were studied at single cell resolution by single cell qPCR. Consistent with the observations in EpiSC populations, the single cell data demonstrated that about 30% of cells in T-EpiSCs and ESD-EpiSCs are T-positive, accompanied by the high expression of the pluripotent marker gene Oct4 and the epiblast marker gene

FIGURE 5. BMP4 promotes mesendoderm lineage commitment through Snail in T-positive EpiSCs and accelerates epidermis fate differentiation via Id1 in T-negative cells. A, Western blotting analysis for Id1 and Snail proteins after CRISPR-Cas9-mediated gene editing of Id1 or Snail in EpiSCs. Expression profiling of pluripotent marker genes Oct4 and Nanog and epiblast marker genes Fgf5 and Nodal of control T-EpiSCs, iCas9-Id1-1/2 T-EpiSCs, and iCas9-Snail-1/2 T-EpiSCs was analyzed by qPCR. B, expression levels of germ layer genes in T-positive cells or T-negative cells FACS-sorted from control T-EpiSCs, iCas9-Id1-1/2 T-EpiSCs, and iCas9-Snail-1/2 T-EpiSCs, treated with or without BMP4 for 24 h in CDM, were analyzed by qPCR. Error bars, S.E.
Fgf5 (Fig. 1), suggesting that the majority of T$^+$ cells are pluripotent cells. Next, T$^+$ and T$^-$ cells were isolated from T-GFP EpiSC by FACS. However, the status of a homogeneous T-GFP-positive population or T-GFP-negative population of EpiSCs could not be maintained. Instead, the dynamic equilibrium and heterogeneity of T-GFP-positive cells and T-GFP-negative cells are readily detected in the EpiSC culture (Fig. 1). Consistently, Tsakirids et al. (30) has also observed a similar phenomenon, and the elevation of Wnt signaling activity may promote a primitive streak-like subpopulation in EpiSCs. However, whether and how the T$^+$ / T$^-$ EpiSCs respond to other extracellular signals to acquire specific lineage fate commitment, even at the single cell level, have not been resolved.

During early mouse gastrulation, extrinsic signals direct pluripotent epiblast cells to give rise to three germ layers. BMP signaling, which is essential to ensure the appropriate embryo patterning in vivo, prevents the neural conversion and promotes the epidermal differentiation and the primitive streak formation (35, 55–57). In mouse EpiSCs in vitro, BMP signaling is imperative for mesendodermal and epidermal cell differentiation (9, 12). The treatment of BMP4 for <12 h can induce the expression of the mesendoderm marker genes in the T$^+$ group but enhance the expression of the epidermis marker genes in the T$^-$ cells (Fig. 3). Furthermore, the differentiation potential of the subpopulations was also evaluated under the same environment for 2 days. T$^+$ cells were more likely to convert to

**FIGURE 6.** T is critical for EMT process during EpiSC mesendoderm differentiation. A, expression levels of T and EMT-related genes in control and T-knockdown EpiSCs in unbiased differentiation at day 4 were determined by qPCR, including epithelial marker gene E-cadherin, mesenchymal marker genes N-cadherin and fibronectin, and transcriptional factors Snail and Zeb2. B, Western blotting analysis of T, E-cadherin, N-cadherin, fibronectin, Snail, phospho-ERK (p-ERK), and ERK in control and T-knockdown EpiSCs during 3 days of unbiased differentiation process. D, day; C, control; KD, knockdown. C, expression levels of mesoderm marker genes Flk1 and Mixl1 and endoderm marker genes Gata6 and Sox17 in control and T-knockdown EpiSCs during 4 days of unbiased differentiation were determined by qPCR. D, immunocytochemical assays (a) and statistical analysis (b) of T, Flk1, and Gata6, in day 3 EBs described in C. Scale bar, 75 μm. Error bars, S.E.
Interestingly, in a T-EpiSC stock culture, neither the repression of BMP signaling by BMP inhibitors, such as Dorsomorphin or Noggin, nor the activation of BMP signaling by BMP4 had an effect on the consistent percentages of T-GFP expression cells (data not shown), indicating that the different responses of T+/T- cells to BMP signaling occur only during the differentiating process of EpiSCs. Further mechanistic study revealed that Snail modulates T+ cells differentiation into mesendoderm lineage upon BMP4 simulation (Fig. 5). Id1, which is a downstream target gene of BMP signaling and plays important roles in the epidermal development (45), participates in the regulation of the conversion from T- cells to the epidermis after BMP4 treatment (Fig. 5). These findings provide evidence of how the interchangeability of EpiSCs contributes to diverse differentiating responses upon BMP signaling at the molecular level.

T is a crucial transcription factor that participates in the primitive streak formation and the mesendoderm lineage commitment in the early mouse embryo in vivo (32, 33, 48). T also plays an essential role in the mesendoderm differentiation of the pluripotent stem cell in vitro (58, 59). Determination of the mesendoderm lineage in early mouse embryo is always associated with the EMT process, during which epithelial epiblast cells lose apical-basal polarity and intercellular junctions (7, 50). Several studies have shown that T promotes EMT in cancer progression and metastasis (60–63). However, so far, whether and how T is involved in EMT process during pluripotent stem cell differentiation, particularly EpiSC differentiation, are still elusively unclear. Here, we uncover that T is functionally involved in promoting EMT by repressing E-cadherin as well as enhancing the expression of Snai1, N-cadherin, and fibronectin (Fig. 6).

Given that the epiblast of the early embryo is undergoing rapid cell proliferation and dramatic molecular and cell fate changes, the findings in our study shed new light on the understanding of how the heterogeneity caused by the oscillatory expression of transcription factors may contribute to the maintenance of self-renewal and the generation of the distinct potency of the pluripotent cells responding to various environmental cues. The heterogeneity in expression of T in EpiSCs may also provide a glimpse into the complex status of the epiblast during early mouse embryo development, indicating that the gastrulation process is dynamic with different progenitors possessing distinct predisposed potentials.

**Experimental Procedures**

**Derivation of T-EpiSCs and Cell Culture**—EpiSCs lines with T-GFP knocked in were established as described previously (12). Briefly, T-GFP ESC (31) aggregates were dissociated into individual single cells and seeded onto FBS-coated dishes. The cells were cultured in CDM supplemented with 12 ng/ml bFGF (Invitrogen) and 20 ng/ml Activin A (R&D Systems) (14). After 6 days, the surviving compact colonies (referred as T-EpiSCs) were dissected into smaller clumps using 2 mg/ml collagenase IV (Invitrogen). T-EpiSCs were routinely passaged at 3-day intervals with medium changed on each day. T-GFP ESCs were cultured in standard ESC culture conditions as reported (31).

**RNA Preparation and qPCR Analysis**—Total RNA was prepared using TRIzol reagent (Invitrogen). Two micrograms of RNA was reverse-transcribed using SuperScript III reverse.

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**FIGURE 7. Model for dynamic heterogeneity of T expression in mouse epiblast stem cells.** T-EpiSCs are pluripotency cells consisting of distinct subpopulation, each of which expresses different levels of the transcription factors T. These two subpopulations are continuously converting into each other. These subsets grossly recapitulate different stages between the egg cylinder epiblast cells (E5.5) and pregastrula cells (E6.5). Undifferentiated T-EpiSC (mostly cells express Oct4 (purple)) cultures contain two major mutually exclusive subpopulations. The T low subpopulation is likely to commit to ectoderm lineages, including epidermis and neural cells. The high T expression populations are primitive streak-biased pluripotent EpiSCs resembling regional restricted mesendoderm. BMP4 could promote mesendoderm lineage commitment through Snail in T+ EpiSCs and accelerate epidermis fate differentiation via Id1 in T- cells.

**Ectoderm**

**BMP4**

**Mesendoderm**

**EpiSC Oct4+**

**T**

**Id1**

**Snail**

**T-**

**T+**

**E5.5**

**E6.5**

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transcriptase (Invitrogen). qPCR was performed with JumpStart TaqReady Mix (Sigma-Aldrich). Each sample was analyzed in triplicate, with the Ct values averaged and then normalized to GAPDH. Primer sequences are available upon request.

**Single Cell PCR**—Single cell PCR was performed as described in the Smart-seq2 protocol (64). Briefly, a trypsin-dissociated single cell was picked by mouth pipette and then lysed in 1 μl of anchored oligo(dT) primer (10 μM), 1 μl of dNTP mix (2.5 mM), 0.2 μl of 10% Nonidet P-40, 0.05 μl of RNase inhibitor (40 units/μl), and 1.2 μl of nuclease-free water at 72 °C for 3 min and immediately placed on ice. 6.55 μl of the reverse transcription mix, containing SuperScript II reverse transcriptase (200 units/μl; Invitrogen), RNase inhibitor, Betaine (5M; Sigma), MgCl2 (100 mM), and locked nucleic acid template switching oligonucleotides (10 μM) were added to each sample, and reverse transcription was performed accordingly. After that, cDNA was amplified by KAPA HiFi Hotstart ReadyMix (KAPA Biosystems) and IS-PCR primer for 19 cycles.

**Immunoﬂuorescence Analysis**—Immunocytochemistry was performed as described (65). The following primary antibodies were used: mouse monoclonal antibodies anti-Oct4 (1:200; Santa Cruz Biotechnology, Inc.) and anti-N-cadherin (1:200; Invitrogen); rabbit polyclonal antibody anti-Nanog (1:100; Santa Cruz Biotechnology); and goat polyclonal antibodies anti-Brachyury (1:100; R&D Systems) and anti-Gata6 (10 g/ml; R&D Systems).

**Flow Cytometry and Cell Sorting**—T-EpiSCs were dissociated into single cell suspension by incubation with trypsin (0.25%)–EDTA (Invitrogen) for 2 min and stained with anti-SEAP1 antibody (1:100; Santa Cruz Biotechnology). The cells were acquired by using an LSRII or FacsCalibur flow cytometer (BD Biosciences) or sorted on a FACSAria (BD Biosciences) FACSort, and the analysis was performed with FlowJo software (Tree Star, Ashland, OR).

**CRISPR/Cas Mediates Loss of Function of Genes in EpiSCs**—To minimize off-targeting effects, the CRISPR design tool was used (Genome Engineering 3.0). Single gRNA was generated by PCR with the primers listed in Table 1.

All four single gRNAs were cloned into the pX330-mCherry plasmid (46, 66). After 48 h of plasmid transfection, the T-EpiSCs were harvested and subjected to FACS. The T-EpiSCs that expressed red fluorescent protein were expanded and passaged in CDM/AF medium. The iCas9-Id1-1, iCas9-Id1-2, iCas9-Null1, and iCas9-Null2 T-EpiSCs cell lines were established (67).

**Gene Knockdown in EpiSCs**—siRNAs were introduced into cells according to the manufacturer’s instructions. For gene knockdown in EpiSCs, mouse T shRNAs were cloned into the lentiviral vector pSilencer-EGFP. Lentivirus packaging and transfection were performed as described (68). GFP-positive cells were sorted with a FACS Aria cell sorter (BD Biosciences). The control and shRNA target sequences are listed in Table 2.

**Whole-mount in Situ Hybridization**—Whole-mount in situ hybridizations were performed as described previously (69). The probes for Oct4, Nanog, and Brachyury were PCR-amplified from mouse cDNA. The PCR primers are listed in supplemental Table S1.

**Western Blotting**—Immunoblotting was performed as described previously (70). The following antibodies were used: anti-phosphorylated Smad1/5/8 (1:2000; Cell Signaling Technology), anti-Smad1 (1:2000; Cell Signaling Technology), anti-phosphorylated ERK1/2 (1:1000; Cell Signaling Technology), anti-ERK1/2 (1:2000; Santa Cruz Biotechnology), anti-T (1:1000; R&D Systems), anti-β-actin (1:7000; Abgene), anti-β-catenin (1:1000; Abcam), anti-Id (1:1000; Abcam), anti-N-cadherin (1:2000; Invitrogen), anti-E-cadherin (1:1000; Santa Cruz Biotechnology), anti-Fibronectin (1:1000; Abcam), and anti-β-catenin (1:1000; Santa Cruz Biotechnology).

**Statistics**—Each experiment was performed at least three times, and similar results were obtained. The data are presented as the mean ± S.D. Student’s t test was used to compare the effects of all treatments. Statistically significant differences are shown as *: p < 0.05; **: p < 0.01.

**Author Contributions**—N. J. and L. S. conceived and designed the study. L. S. performed most of the experiments. J. C. performed the single cell analysis. N. J. and L. S. analyzed the data. L. S., K. T., G. P., and N. J. wrote the manuscript. All authors approved the final version of the manuscript.

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