The Roles of Testicular C-kit Positive Cells in De novo Morphogenesis of Testis

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C-kit positive (c-kit+) cells are usual tissue-specific stem cells. However, in postnatal testis, undifferentiated spermatogonical stem cells (SSCs) are c-kit negative (c-kit−) and activation of c-kit represents the start of SSC differentiation, leaving an intriguing question whether other c-kit+ cells exist and participate in the postnatal development of testis. To this end, a feasible system for testicular reconstitution, in which a specific type of cells can be manipulated, is needed. Here, we first establish de novo morphogenesis of testis by subcutaneous injection of testicular cells from neonatal testes into the backs of nude mice. We observe testicular tissue formation and spermatogenesis from all injected sites. Importantly, functional spermatids can be isolated from these testicular tissues. Using this system, we systematically analyze the roles of c-kit+ cells in testicular reconstitution and identify a small population of cells (c-kit+:CD140a+:F4/80−), which express typical markers of macrophages, are critical for de novo morphogenesis of testis. Interestingly, we demonstrate that these cells are gradually replaced by peripheral blood cells of recipient mice during the morphogenesis of testis. Thus, we develop a system, which may mimic the complete developmental process of postnatal testis, for investigating the testicular development and spermatogenesis.

It is well known that activation of c-kit signaling is critical in cell migration, survival, proliferation, self-renewal and differentiation. Expression of c-kit has been used as a marker for isolating tissue-specific stem cells or progenitor cells, such as hematopoietic stem cells/progenitor cells, cardiac stem cells and lung stem cells. Interestingly, bone marrow-derived c-kit+ cells could promote cardiac repair by stimulation of the activity of endogenous cardiac progenitor cells, indicating that c-kit+ cells play key roles in tissue development and regeneration.

Testicular development is a complex process that can be roughly divided into embryonic and postnatal stages. During fetal gonadal development, the expression of c-kit regulates migration, survival and proliferation of primordial germ cells (PGCs). The male PGCs become arrested at the G0/G1 of the cell cycle at around 13.5 days post-coitum and begin to divide mitotically again around 3 days after birth during which the expression of c-kit is dramatically reduced. Reactivation of c-kit in postnatal testis is detected in differentiating SSCs, but not in undifferentiated SSCs. Furthermore, from c-kit− cell population, SSCs can be highly enriched using several other surface markers. Taken together, activation of c-kit is not required for SSC self-renewal, but for spermatogenesis. This leaves an open question of whether other c-kit+ cells exist and play important roles in postnatal development of testis.

In past decades, a variety of model systems have been developed to recapitulate spermatogenesis and testicular development in vitro and in vivo. However, a feasible system for studying the function of different cells in testicular development is lacking. An emerging technique, in which, testicular cell-derived tissue (TCDT) can be de novo formed after ectopic transplantation of cells dissociated from newborn testes into the subcutis of
immunodeficient mice, could mimic the complete process of postnatal testicular development. This technique, termed *de novo* morphogenesis of testis19, has been used to reconstitute mouse20, rat20, porcine20 and sheep22 testes in immunodeficient mice. One intriguingly potential application of this approach is to manipulate different cells prior to grafting20, providing an opportunity to reveal the function of different cells in postnatal development of testis. However, the efficiency of spermatogenesis in these *de novo*-formed TCDTs is low20,21, and the functional male gametes could be generated only by integration of exogenous SSCs20, restricting its broad range of applications for studying spermatogenesis and testicular development.

In this study, we modified the process of grafting testicular cells and obtained TCDTs from all transplants and found that spermatogenesis happened in all tissues. Importantly, functional spermatids could be isolated from all TCDTs. Using this approach and fluorescence-activated cell sorting (FACS), we systemically analyzed the role of *c-kit* cells in postnatal development of testis. We identified that a small population of cells (*c-kit*:CD140a⁻:F4/80 sym), which express typical markers of macrophages, were critical for *in vivo* testicular reconstitution.

**Results**

**Functional spermatogenesis established in all testicular cell-derived tissues (TCDTs) from transplants without Matrigel Matrix (MGM).** To establish the system of *de novo* morphogenesis of testis, we modified a protocol that was previously reported by Kita et al20. Briefly, testes of 5.5–6.5 days old male mice (B6D2F1 background) were decapsulated and digested into single cells. The cell suspension mixed with (the group 2), as reported by Kita et al20, or without (the group 1) same volume of Matrigel Matrix (MGM), with a final concentration of 1 × 10⁶ cells/ml, was injected subcutaneously into the backs of nude mice. A total of 1 × 10⁶ cells (100 µl of cell suspension) were injected for each transplant. Three months later, we observed tissue formation from all grafted cells with or without MGM (5 and 8, respectively) (Supplementary Fig. 1a). Interestingly, the average weight of the TCDTs formed from the group 2 was significantly higher than that from the group 1 (Supplementary Fig. 1b). Histological analyses indicated, however, the presence of seminiferous tubular-like structures in all tissues from the group 1 while only a few tubules existed in the group 2 (Fig. 1a). Immunostaining of GATA-1, Cyp17 and α-smoothmuscle actin (SMA), specific markers for mature sertoli cells, Leydig cells and myoid cells respectively, showed that a large number of typical testicular cords existed in TCDTs from the group 1 while only a few tubules existed in the group 2 (Fig. 1a). Testicular cords existed in TCDTs from all transplants in nude mice (referred as functional TCDTs to distinguish them from those from group 2), implying that this system may be feasible for investigating the roles of different cells in postnatal development of testis.

**The role of SSCs in TCDT formation.** SSCs are the only known stem cells in adult testis that maintain self-renewal and spermatogenesis20. We next assessed the role of SSCs in TCDT formation. For this purpose, the endogenous SSCs were largely removed from testicular cells according to two cell surface markers, *c-kit* and CD9. *C-kit* is a marker for differentiating spermatogonia14 and CD9 is a common surface marker for murine germ line cells24. *C-kit* negative and CD9 positive (*c-kit*:CD9⁺) cells (P4 zone in Fig. 2a) presented enriched SSC population, demonstrated by highly expression of Oct427 and GFRα128 (Fig. 2b), another two SSC markers. After depletion of *c-kit*:CD9⁺ cells, the rest of testicular cells (P6 zone in Fig. 2a) were injected subcutaneously into the backs of nude mice at a concentration of 1 × 10⁶ cells/ml (100 µl of cell suspension per injection). We examined TCDT formation three months later and found that all grafts formed tissues (9 of 9) (Supplementary Fig. 2a). The TCDT from P6 zone cells had smaller size and lower weight than those of control TCDTs (Supplementary Fig. 2b). Immunostaining analysis showed that typical testicular cords existed in all TCDTs from P6 zone cells (Fig. 2c); however, germ cells could rarely be observed in these cords (Fig. 2d).

Next, we tested whether addition of exogenous SSCs in P6 zone cells could reestablish spermatogenesis in TCDTs. To this end, we first generated SSC line by *in vitro* culturing *c-kit*:CD9⁺ cells isolated from testes of *actin-EGFP* transgenic male mice (heterozygote) according to the protocol described by Kanatsu-Shinohara et al29. The established SSC line (termed EGFP-SSC) showed typical SSC morphology (Supplementary Fig. 3a, b) and maintained paternal genomic imprints (Supplementary Fig. 3c). Moreover, EGFP-SSCs could restore spermatogenesis after transplantation into the testes of busulfan-treated male mice (Supplementary Fig. 3d), resulting in generation of functional male gametes (Supplementary Fig. 3e and Table 1). We then mixed the EGFP-SSCs with wide-type (WT) P6 zone cells at a ratio of 1:1 and transplanted into the backs of nude mice. Functional TCDTs were formed from all grafts (3 of 3) and the weight of TCDTs was resumed and even higher than that of control TCDTs, probably due to the higher ratio of SSCs in injected cells than that in control cells (Supplementary Fig. 2a, b). Importantly, the EGFP-SSC recolonized in seminiferous cords derived from WT P6 zone cells and generated EGFP-SSC-derived spermatogenesis (Fig. 2e, f). Flow analysis indicated that TCDTs contained around 5.5% of haploid cells, 41.6% of which were EGFP positive (Supplementary Fig. 2c), reflecting the donor SSCs carried heterozygous EGFP transgene. ROSI examinations demonstrated that spermatids...
isolated from TCDTs were competent for fertilization to produce the progeny (Fig. 2g and Table 1). Taken together, these data validates the idea that the system of de novo morphogenesis of testis is feasible for analyzing the function of different cells in postnatal development of testis.

Interstitial c-kit<sup>1</sup> cells (c-kit<sup>1</sup>:CD140a<sup>1</sup> cells) are critical for the TCDT formation. We next investigated the role of c-kit<sup>1</sup> cells in TCDT formation. As shown in Fig. 3a, around 3.8% of testicular cells dissociated from testes of 6.5 days old mice (WT mice, Fig. 3a left and EGFP mice, Fig. 3a right) were c-kit<sup>1</sup> cells. After transplantation of c-kit<sup>1</sup> cells into nude mice, only 2 of 20 grafts formed TCDTs with very small size, indicating that c-kit<sup>1</sup> cells were critical for TCDT formation (Fig. 3b, c). To further verify the vital role of c-kit<sup>1</sup> cells in TCDT formation, c-kit<sup>1</sup> cells from EGFP-marked testes were mixed with c-kit<sup>2</sup> cells from WT mice (B6D2F1) for subcutaneous injection (Fig. 3a). As expected, all three grafts formed TCDTs with similar size to control TCDTs (Fig. 3b, c). Immunostaining analysis demonstrated that seminiferous cords and spermatogenesis existed in all three TCDTs (Fig. 3d). Interestingly, most EGFP positive cells were of c-kit<sup>2</sup> origin.
located in interstitium of TCDTs and didn’t express Cyp17 (Fig. 3d); while a small number of EGFP1 cells were observed in seminiferous cords and expressed MVH (Fig. 3e). Because c-kit1 cells could be roughly divided into two fractions according to their light-scattering features (Supplementary Fig. 4a), we proposed that two subpopulations of c-kit1 cells, which produced progeny cells distribution in interstitium and seminiferous cords respectively, exist in postnatal testes.

We attempted to separate c-kit1 cells into two subpopulations on the basis of surface markers. Flow analyses showed that CD140a could separate c-kit1 cells into two groups (Fig. 4a and Supplementary Fig. 4b, c). Immunostaining analysis of postnatal testis

| Origin of injected oocytes | No. of Injected oocytes | No. of 2-cell | Embryo Stage | No. of Embryos Transferred | No. of recipients | Number of Pups (% of Transferred Embryos) | Number of Pups Surviving to Adulthood |
|---------------------------|-------------------------|--------------|--------------|----------------------------|-------------------|------------------------------------------|----------------------------------------|
| TCDTs from F1 mice       | 86                      | 72           | Blastocyst   | 16                         | 1                 | 2 (12.5)                                 | 1                                       |
| EGFP-SSCs Reconstituted testes | 150                | 150          | two-cell     | 60                         | 2                 | 4 (6.7)                                  | 4                                       |
| TCDTs from P6 zone cells plus EGFP-SSCs | 114                | 112          | blastocyst   | 36                         | 2                 | 5 (13.8)                                 | 4                                       |
| C-Kit1 EGFP-SSCs Reconstituted testes | 135               | 134          | two-cell     | 60                         | 2                 | 6 (10)                                   | 6                                       |
| TCDTs from F1 c-Kit1 plus EGFP c-kit1::CD140a cells | 68                 | 60           | two-cell     | 40                         | 2                 | 5 (12.5)                                 | 2                                       |
| TCDTs from F1 c-Kit1 plus EGFP F4/80t cells | 120               | 105          | two-cell     | 75                         | 3                 | 13 (17.3)                                | 13                                      |
| F1 mice                  | 52                      | 48           | blastocyst   | 12                         | 1                 | 3 (25)                                   | 3                                       |

* RS, round spermatids.

Figure 2 | The role of SSCs in TCDT formation. (a) C-kit negative and CD9 positive (c-kit1::CD91) cells were enriched from testicular cells of neonatal mice via FACS. C-kit negative and CD9 positive (c-kit1::CD91) cells (P4 zone) presented enriched SSC population. (b) C-kit1::CD91 cells (P4 zone cells) highly expressed Oct4 and GFRα1, another two SSC markers. The expression levels were relative to those in established SSC line, which were set to 1. Results were from three independent cell sorting. Values are means ± SD. (c) Immunostaining analysis of TCDT formed from P6 zone cells (testicular cells without P4 zone cells). Typical testicular cords, which contain a large amount of Cyp17 positive cells (red fluorescence) and SMA positive cells (green fluorescence), exist in all TCDTs from P6 zone cells. The nuclei were stained with DAPI (blue fluorescence). Scale bar, 50 µm. (d) Summary of the ratio of cords with germ cells in total cords in TCDTs formed from testicular cells (n = 3) and P6 zone cells (n = 5). Germ cells can be rarely observed in the cords of TCDTs formed from P6 zone cells. Values are means ± SD. ** 0.001 < P < 0.01. (e) Image of the TCDT formed from WT P6 zone cells plus exogenous EGFP-marked SSCs. EGFP-positive cells can be observed in entire TCDT. Scale bar, 2 mm. (f) Immunostaining analysis of TCDT formed from WT P6 zone cells plus exogenous EGFP-marked SSCs. All EGFP-positive cells express Mvh, indicating that EGFP-SSCs recolonize in seminiferous cords derived from WT P6 zone cells and generate EGFP-SSC-derived spermatogenesis. The nuclei were stained with DAPI. Scale bar, 50 µm. (g) FACS-enriched haploid cells could support in vitro and in vivo development after injection into oocytes (ROSI). Left, blastocysts generated from ROSI experiments. Some of them are EGFP positive, reflecting that donor SSCs carried heterozygous EGFP transgene. Right, the newborn pups from ROSI experiments. Scale bar, 100 µm.
indicated that, consistent with previous observations16,30, CD140a (also known as platelet-derived growth factor ralpha, PDGFR) was specifically expressed in interstitial cells and c-kit was expressed both in the seminiferous and interstitial cells (Fig. 4b). Furthermore, FACS-enriched c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells, but not c-kit:\textsuperscript{2} CD140a\textsuperscript{2} cells, expressed MVH (Fig. 4c) and also CD49f (also known as integrin\textsuperscript{26}) (Supplementary Fig. 4d), another well-known surface marker for mouse germ cell. Finally, in vitro culture experiments showed that c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells did not expand in SSC culture environment (Supplementary Fig. 4e, left). In contrast, some c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells proliferated (Supplementary Fig. 4e, right) and formed a functional SSC line (termed c-kit:\textsuperscript{1} EGFP-SSC) (Supplementary Fig. 5a–d). This is consistent with a previous report that differentiating SSCs can be converted into undifferentiating state in vitro and in vivo\textsuperscript{21}, supporting our early observations that a small amount of EGFP-marked c-kit\textsuperscript{1} cells recolonized in seminiferous tubules of TCDTs (Fig. 3e). Taken together, c-kit\textsuperscript{1} is expressed both in interstitial and seminiferous cells and CD140a\textsuperscript{1} is a suitable surface marker for distinguishing two subpopulations of c-kit\textsuperscript{1} cells.

We next tested the roles of interstitial and seminiferous c-kit\textsuperscript{1} cells in TCDT formation. After injection of cell suspensions containing WT c-kit\textsuperscript{1} cells and EGFP-marked c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells into nude mice, all transplants (7 of 7) formed TCDTs with comparable weight to controls three months later (Fig. 4d). In contrast, c-kit:\textsuperscript{2} CD140a\textsuperscript{2} cells did not rescue the failure of TCDT formation induced by c-kit\textsuperscript{2} cells depletion (3 of 6 formed TCDTs with small size, Fig. 4d). We found that integral seminiferous cords and spermatogenesis existed in all TCDTs derived after reimbursement of exogenous c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells in c-kit\textsuperscript{1} cells (Fig. 4e). Haploid cells (Fig. 4f) were enriched by FACS and competent for fertilization (Fig. 4g and Table 1). Together, interstitial c-kit\textsuperscript{1} cells (c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells) alone can rescue the failure of TCDT formation induced by depletion of c-kit\textsuperscript{2} cells from testicular cells.

**A small subpopulation of interstitial c-kit\textsuperscript{1} cells (C-kit\textsuperscript{1} CD140a\textsuperscript{1} F4/80\textsuperscript{+} cells) determine the TCDT formation.** The constitutively expressed EGFP in donor cells provides a system for lineage tracing of individual cell population in TCDT formation. Green-fluorescence cells, which were the progeny cells of EGFP-marked c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells were all located in interstitium (Fig. 4e). Interestingly, these cells didn’t express Cyp17 (Fig. 4e), consistent with our early observations in Fig. 3d, indicating that c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells do not include the progenitor cells of adult Leydig cells. Because Leydig cell is the only known interstitial cell that expresses c-kit by now\textsuperscript{32,33}, these results imply that different types of c-kit\textsuperscript{1} cells exist in interstitium and play important role in TCDT formation. To test this hypothesis, we enriched c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells of testis from 6.5 days old mice by FACS. Immunostaining analysis showed that around 50% of c-kit\textsuperscript{1} CD140a\textsuperscript{1} cells expressed Cyp17 (Fig. 5a). Because progeny cells of exogenous c-kit:\textsuperscript{1} CD140a\textsuperscript{1} cells in TCDTs didn’t express Cyp17 (Fig. 3d and Fig. 4e), we concluded that these c-kit:\textsuperscript{1} CD140a\textsuperscript{1} Cyp17\textsuperscript{+} cells were fetal Leydig cells, which persisted in postnatal interstitium and were eventually replaced by adult Leydig cells in TCDT, consistent with the observations in normal testicular development\textsuperscript{11}. 

**Figure 3 | The role of c-kit\textsuperscript{1} cells in TCDT formation.** (a) WT c-kit\textsuperscript{1} cells (P3 zone cells in left picture) plus EGFP-c-kit\textsuperscript{1} cells (P2 zone cells) were injected into nude mice for TCDT formation. (b) Summary of TCDT formation. After depletion of c-kit\textsuperscript{2} cells from testicular cells of neonatal testes, only 2 of 20 grafts of c-kit\textsuperscript{2} cells formed TCDTs. After reimbursement of EGFP-c-kit\textsuperscript{1} cells from neonatal testes into WT c-kit\textsuperscript{1} cells, all 3 grafts formed TCDTs. (c) The weight of TCDTs. TCDT from c-kit\textsuperscript{2} cells had a smaller size than controls. After reimbursement of EGFP-c-kit\textsuperscript{1} cells, TCDT could be formed with similar size to control TCDTs (n = 5, 2 and 3 for each group, respectively). Values are means ± SD. "n.s." means no significant difference, ***P = 0.0001. (d) Immunostaining of Mvh and Cyp17 in sections of TCDTs from the group of WT c-kit\textsuperscript{1} cells plus EGFP-c-kit\textsuperscript{1} cells. Most EGFP positive cells located in interstitium of TCDTs and didn’t express Cyp17. Scale bar, 50 μm. (e) Immunostaining of the same section as (d). Yellow arrowheads indicate that there are a small number of EGFP+ cells in seminiferous cords, which express MVH. Scale bar, 50 μm.
We next attempted to identify what the c-kit<sup>+</sup>:CD140a<sup>+</sup> cells are. It is well known that macrophages comprise a substantial portion of interstitial cells of testis<sup>34,35</sup>. We then performed flow analysis according to F4/80, a surface marker of macrophages<sup>36,37</sup>. As expected, we found around 0.35% of testicular cells from 6.5 days old pups were F4/80 positive cells and all these cells expressed c-kit (Fig. 5b). Furthermore, c-kit<sup>+</sup>:CD140a<sup>+</sup> cells could be divided into two subpopulations according to F4/80 antibody (Fig. 5c). Flow analysis showed that all the F4/80 positive cells in the neonatal testes were also CD11b positive, another well-known surface marker of macrophage<sup>38</sup>. Furthermore, real-time PCR analysis showed that c-kit<sup>+</sup>:CD140a<sup>+</sup>:F4/80<sup>+</sup> (termed F4/80<sup>+</sup>) cells highly expressed transforming growth factor-β (TGFβ)<sup>39,40</sup>, a secretory protein of testicular macrophages, while c-kit<sup>+</sup>:CD140a<sup>+</sup>:F4/80<sup>−</sup> (termed F4/80<sup>−</sup>) cells expressed Cyp17 and 3βHSD, specific markers for adult Leydig cells<sup>41</sup> (Supplementary Fig. 6a).

In vitro culturing experiments showed that F4/80<sup>+</sup> cells could expand in Macrophage Complete Medium (MCM)<sup>38</sup> (Supplementary Fig. 6b), but could not in a standard culture medium for mouse embryonic fibroblasts (MEF). By contrast, F4/80<sup>−</sup> cells could be maintained in MEF medium (Supplementary Fig. 6c). Functional analyses showed that F4/80<sup>+</sup> cells inhibited the proliferation of T cells (Supplementary Fig. 6d<sup>42</sup>) and promoted the testosterone secretion by F4/80<sup>−</sup> cells in vitro (Supplementary Fig. 6e<sup>43</sup>). Taken together, these data demonstrate that F4/80<sup>−</sup> cells are most likely macrophages and F4/80<sup>+</sup> cells mostly comprise fetal Leydig cells.

We then analyzed the roles of F4/80<sup>−</sup> and F4/80<sup>+</sup> cells in TCDT formation. After injection of cell suspensions containing WT c-kit<sup>+</sup>:CD140a<sup>+</sup> cells alone (n = 7), but not c-kit<sup>+</sup>:CD140a<sup>−</sup> cells (EGFP marked) (n = 6), could rescue the failure of TCDT formation induced by c-kit<sup>−</sup> cell depletion. Right, the average weight of TCDTs (n = 4) formed from c-kit<sup>−</sup> cells plus exogenous c-kit<sup>−</sup>:CD140a<sup>−</sup> cells were significantly higher than that of TCDTs (n = 3) from c-kit<sup>−</sup> cells plus exogenous c-kit<sup>−</sup>:CD140a<sup>−</sup> cells. Values are means ± SD, *** P < 0.001.

(e) Immunostaining analysis of TCDT from the group of WT c-kit<sup>−</sup> cells plus EGFP-marked c-kit<sup>−</sup>:CD140a<sup>−</sup> cells using Mvh and Cyp17 antibodies. Integral seminiferous cords and spermatogenesis exist in the TCDTs, indicating that these are functional TCDTs. EGFP positive cells, which are progeny cell of donor c-kit<sup>−</sup>:CD140a<sup>−</sup> cells, locate in interstitium and don’t express Cyp17, reflecting that c-kit<sup>−</sup>:CD140a<sup>−</sup> cells are not progenitors of Leydig cells. Scale bars, 50 μm. (f) Haploid cells could be enriched from TCDTs formed from WT c-kit<sup>−</sup> cells plus EGFP-marked c-kit<sup>−</sup>:CD140a<sup>−</sup> cells through FACS. (g) Newborn pups developed from reconstituted oocytes after injection of haploid cells enriched from TCDT showed in (f).

Figure 4 | Interstitial c-kit+ (c-kit<sup>+</sup>:CD140a<sup>+</sup>) cells are critical for the TCDT formation. (a) Flow analysis showed that CD140a could separate c-kit<sup>+</sup> cells of neonatal testes into two groups (P6 zone and P7 zone). (b) Immunostaining analysis of postnatal testis indicated that CD140a (red fluorescence) was specifically expressed in interstitial cells and c-kit (green fluorescence) was expressed both in the seminiferous and interstitial cells. Green box in left image is magnified in right. Scale bars, 25 μm. (c) Immunostaining of FACS-enriched c-kit<sup>+</sup>:CD140a<sup>+</sup> cells and c-kit<sup>+</sup>:CD140a<sup>−</sup> cells using CD140a and Mvh antibodies. Left, c-kit<sup>+</sup>:CD140a<sup>+</sup> cells don’t express Mvh. Right, c-kit<sup>+</sup>:CD140a<sup>−</sup> cells express Mvh. Scale bar, 50 μm.

(d) Summary of TCDT formation. Left, interstitial c-kit<sup>+</sup> (EGFP-marked c-kit<sup>+</sup>:CD140a<sup>+</sup>) cells alone (n = 7), but not c-kit<sup>+</sup>:CD140a<sup>−</sup> cells (EGFP marked) (n = 6), could rescue the failure of TCDT formation induced by c-kit<sup>−</sup> cell depletion. Right, the average weight of TCDTs (n = 4) formed from c-kit<sup>−</sup> cells plus exogenous c-kit<sup>−</sup>:CD140a<sup>−</sup> cells were significantly higher than that of TCDTs (n = 3) from c-kit<sup>−</sup> cells plus exogenous c-kit<sup>−</sup>:CD140a<sup>−</sup> cells. Values are means ± SD, *** P < 0.001.
cells (one million cells) and EGFP-marked F4/80+ cells (25 thousand cells) into nude mice, all transplants (5 of 5) formed TCDTs with comparable weight to controls three months later (Fig. 5d). In contrast, F4/80− cells did not rescue the failure of TCDT formation induced by c-kit+ cells depletion (2 of 7 formed TCDTs with small size, Fig. 5d). Haploid cells (Fig. 5e) could be enriched from TCDTs and support full-term development of injected oocytes (Fig. 5f and Table 1). Together, F4/80+ cells alone can rescue the failure of TCDT formation induced by depletion of c-kit+ cells from testicular cells.

F4/80+ cells are gradually replaced by cells from recipient mice during TCDT formation. Next, we analyzed the distribution of EGFP-positive cells, which were the progeny cells of EGFP-marked F4/80+ cells from fetal testes. Surprisingly, no EGFP-positive cells could be observed in all TCDTs (Fig. 6a, b), while a significant population of F4/80+ cells existed in all TCDTs (Fig. 6b). To test the origin of TCDT-derived F4/80+ cells, DNA was extracted and employed for simple sequence-length polymorphism (SSLP). The results showed that the TCDT-derived F4/80+ cells had a polymorphic pattern same as that of recipient strain (BALB/C), but different from the strain of injected cells isolated from neonatal testes (B6D2F1, Fig. 6c). In vitro culturing experiments showed that TCDT-derived F4/80+ cells could expand in MCM (Fig. 6d), but could not in a standard culture medium for MEF. Functional analyses showed that TCDT-derived F4/80+ cells could inhibit the proliferation of T cells (Fig. 6e), indicating that TCDT-derived F4/80+ cells, which were originate from peripheral blood of recipient mice, were most likely macrophages. We then analyzed the timing of F4/80+ cells replacement during TCDT formation by dissecting TCDT at 30, 45, 60 days post injection of EGFP-marked testicular cells into nude mice. Cells were dissociated from TCDTs and employed for flow analysis. The results showed that the ratio of total F4/80+ cells gradually increased while EGFP-marked F4/80+ cells gradually reduced (Fig. 6f).

Finally, we investigated whether F4/80+ cells from other sources, including F4/80+ cells from adult testes, peritoneal F4/80+ cells and bone-marrow-cell-derived F4/80+ cells, can be used in place of F4/80+ cells from neonatal testes to rescue the failure of TCDT formation induced by c-kit+ cells depletion. To this end, 25 thousands of F4/80+ cells were mixed with 1 million c-kit+ testicular cells from neonatal mice for each transplantation. Three month later, we didn’t find the TCDT formation from all injection sites (4 from F4/80+ cells from adult testes, 4 from peritoneal F4/80+ cells and 3 from bone-marrow-cell-derived F4/80+ cells).

Taken together, c-kit+:CD140a+:F4/80+ cells from fetal testes are critical for TCDT formation; however, these cells are gradually eliminated and replaced by F4/80+ cells originated from peripheral blood cells of recipient mice.

Discussion

The mammalian testis is complex organ and provides a host to spermatogenesis, one of the most intricate cell divisions occurring in postnatal life. Different cells participate in testicular development and play different roles in spermatogenesis. Great efforts have been made and a variety of model systems have been established since early in 20th century to recapitulate the spermatogenesis in vitro and in vivo. However, none of these systems are feasible for analyzing cellular function in postnatal testicular development and spermatogenesis. De novo morphogenesis of testis, a developing technique, provides a unique method to study testicular development.
since it enables to manipulate various cells prior to grafting\(^a\). However, this application has not yet been tested experimentally. Moreover, the system reported to date shows very low spermatogenesis\(^{20,21}\) in reconstituted tissues, greatly restricting its application. In our study, we modified the system by removing MGM from reported protocol\(^{20}\) and injecting freshly isolated testicular cells into nude mice. We found that all TCDTs were generated from all grafts (48 of 48). Surprisingly, functional spermatogenesis occurred in all TCDTs (48 of 48) and the highest ratio of haploid cells in total cells of TCDTs was 13.3\%. MGM has been used for 3-Dimensional culture of immature testicular cells to support cord formation\(^{47,48}\) and later for in vivo morphogenesis of testis\(^{20}\). Although MGM may help testis-like tissue formation in vitro and in vivo, consistent with significantly higher weight of TCDTs from the group 2 with MGM than that from the group 1 without MGM in this study (Supplementary Fig. 1a), MGM may not be benefit for homing of SSC, resulting in low spermatogenesis in formed tissues as shown in this study (Fig. 1d) and reported previously\(^{20}\).

We develop a new strategy for studying the function of different cells in testicular development by employing the technical combination of cell sorting and de novo morphogenesis of testis. In this system, interested cells in testes are removed and reimbursed with same cells carrying transgene of fluorescent proteins, such as EGFP (Fig. 7a). The reconstituted cell suspensions are used for TCDT formation and the fluorescence-marked cells can be traced in formed tissues. We validate this system by depleting endogenous SSCs and...
addition of exogenous SSCs carrying EGFP transgene (Fig. 2). We further systematically analyze the roles of c-kit− cells in TCDT formation and identify that a small population of c-kit+ cells (c-kit−:CD140a−:F4/80−) from neonatal mice, which are most likely testicular macrophages, play critical role in TCDT formation.

Macrophages are important accessory cells for testicular development and decide the testis as one ‘immunological privileged’ tissue of the body. However, there is no direct evidence that macrophages are critical for testicular development because all reports to date are based on depletion of macrophages by chemical treatment or gene knockout experiments, in which, macrophages can not be completely removed from testes. Meanwhile, it is still not clear the origin of testicular macrophages. One possibility of their origination is that cells from blood move into testis and locally differentiate into macrophages; however, this hypothesis has not been proven yet. Here, we provide the most direct evidence to date that c-kit−:CD140a−:F4/80− cells of neonatal testis, which are most likely macrophages, are critical for testicular development and F4/80− cells of adult testes are most likely derived from peripheral blood cells.

In summary, we have shown several advantages of this new strategy in our study (i) the function of specific type of cells in testicular development can be demonstrated by deletion of these cells from testicular cells for TCDT formation and further confirmed by restoration-of-function experiments, i.e., reimbursement of the same type of cells to the rest of testicular cells; (ii) addition of exogenous cells carrying transgenes provides an opportunity to trace the progeny cells of donor cells in formed TCDTs, allowing us to reveal new progenitor cells of specific cell type, such as adult Leydig stem cells; and (iii) genetic modification in a specific type of cells to the rest of testicular cells.

TCDT formation allows us to elucidate the gene function in testicular development and spermatogenesis.

Methods
Animal use and care. All animal procedures were carried out in accordance with the approved guidelines of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, China.

WT B6D2F1 mice or EGFP-F1 mice were derived from WT or actin-EGFP C57BL/6 female mice after mating with DBA/2J male mice. Nude mouse were purchased from SLACS (Shanghai, China).

Generation of testicular cell-derived tissue (TCDT). Donor cells for transplantation were prepared from testes of WT F1 or EGFP-F1 mice (5.5–6.5 days old). The testes were decapsulated and were digested by a two-step enzymatic treatment. Briefly, testicular tissue was treated with digestion solution I containing collagenase type IV (1 mg/ml) and DNase (150 ug/ml) in DMEM at 37°C for 20 min. The specimens were centrifuged and washed twice with PBS and digested with trypsin at 37°C for 5 min. The cell suspension was filtered through mesh with a pore size of 40 μm. For subcutaneous injection, we counted the cell number using the Countess (Invitrogen).

Preparation of F4/80+ cells from other sources. Peritoneal F4/80+ cells were prepared by intraperitoneal injection of 4% thioglycollate into B2D6F1 mice (8 wks). Three days later, elicited peritoneal F4/80+ cells were harvested by recovering the DMEM medium following injection them into the peritoneal cavity. To obtain bone-marrow-derived F4/80+ cells, bone marrow cells form femurs and tibias of B6D2F1 mice (8 wks) were cultured in DMEM/F12 medium supplemented with 10% FBS and 20 ng/ml M-CSF (ebiSciences) for 7 days.

Cell sorting. The testicular cells were suspended with moderate PBS containing 8% fetal bovine serum (PBS/FBS) and were incubated with antibodies. Flow cytometric analyses were performed using a standard protocol. All the antibody information was listed in Table S1. Antibody incubations were performed on ice for 30 min and cells were washed with PBS/FBS twice. For sorted the haploid cell from the testes and

![Figure 7](https://www.nature.com/scientificreports/srep05936_f7.png)
TCDTs, the cell suspension were incubated with the Hoechst (final concentration 5 ug/ml) and PI (final concentration 2 ug/ml) at 37 °C for 30 min and were washed with PBS/FBS twice. Samples were all sorted on a BD FACS Aria II cell sorter.

**Immunohistochemical analysis.** The tissues were fixed in 4% paraformaldehyde, frozen in Tissue-Freezing medium, sectioned at a thickness of 10 microns. The sections were stained with primary antibody against MIS, GATA-1, γ-SMA, Mvh, CYP17A1 (Cyp17) and c-Kit for one hour at 37 °C. After rinsing, sections were incubated for one hour at 37 °C with a corresponding secondary antibody. All the antibody information was listed in Table S1. The nuclei were counterstained with Hoechst 33342. The frozen sections from normal testis were used as controls. The experiments were repeated for more than three times. The frozen sections from adult testis were set as positive control and negative control for all antibodies. The experiments were repeated more than three times.

**Bisulfite sequencing.** The DNA samples extracted from the round sperms and SSCs were enzymatic digested using the restriction enzyme BamHI. The PCR products corresponding to the putative region were amplified from the DNA samples and cloned into pMD19-T vectors (Takara). The individual clones were sequenced by Invitrogen, Shanghai. Bisulfite primer information is presented in Table S2.

**SSC culture and transplantation.** For deriving EGFP-SSC lines, the sorted cells were seeded on the mouse embryonic fibroblast (MEF) feeder cells and cultured with the modified SSC medium. The culture medium consisted of StemPro-34 SFM (Invitrogen) supplemented with StemPro supplement (Invitrogen), 25 µg/ml insulin, 100 µg/ml transferrin, 60 µM putrescine, 30 mM sodium selenite, 6 mg/ml D(+)-glucose, 30 µg/ml pyruvic acid, 1 µM dl-lactic acid (Sigma), 5 mg/ml bovine serum albumin (Sigma), 2 mM l-glutamine, 2-mercaptoethanol (Millipore), minimal essential medium vitamin solution (Invitrogen), non-essential amino acid solution (Millipore), L-glutamine (Millipore), penicillin/streptomycin (Invitrogen), 0.1 mM ascorbic acid, 10 µg/ml d-Biotin (Sigma), 20 ng/ml recombinant human epidermal growth factor (Invitrogen), 10 ng/ml recombinant human basic fibroblast growth factor (Invitrogen), 10 ng/ml recombinant human granulocyte macrophage colony-stimulated factor (Invitrogen) and 1% fetal bovine serum (ES cell-qualified, Invitrogen). The cells were maintained at 37 °C in an atmosphere of 5% carbon dioxide in air. Culture medium was changed every 2–4 days. All cultured cells were tested to make sure be mycoplasma free. For SSCs transplantation, busulfan (40 mg/kg)-treated 4–6 weeks old F1 male mice were used as the recipient mice. Approximately 2–3 × 10^5 cells were injected with a micropipette (60 µm diameter tips) into the seminiferous tubules of recipient mice through the efferent duct. The mice were sacrificed 2 months later, and the testes were examined under a fluorescence microscope to detect existence of transplanted SSCs by EGFP expression.

**Intracytoplasmic round spermatid injection (ROSI).** Metaphase II-arrested oocytes were collected from superovulated B6D2F1 females (8–10 wks) and cumulus cells were removed using hyaluronidase. Round spermatids and mature sperms were enriched from the TCDTs and reconstituted testes and injected into the oocytes in a droplet of HEPEs/CSM medium containing 5 µg/ml cytchalasin B, 0.5 µg/ml PI, a blunt Pico-driven pipette. The oocytes were then activated by treatment with SrCl2 in a droplet of HEPES-CZB medium containing 5 µM CaCl2. The oocytes were then activated by treatment with SrCl2 in a droplet of HEPES-CZB medium containing 5 µM CaCl2. The fertilized oocytes were cultured for 24 h or 3.5 d and two-cell embryos or postmeiotic cells by Hoechst 33342 in mouse spermatogenesis. **Dores, C., Alpaugh, W. & Dobrinski, I. From in vitro culture to in vivo models to clinical applications. Physiol Rev 92, 1619–1649 (2012).**

**Statistical analysis.** The weight of TCDTs and the ratios of germ cells in TCDTs were analyzed by unpaired Student’s t test. All statistical analyses were done by Graphpad software 5.0. P value > 0.05 was assumed to be not significant (n.s.), P value < 0.05 was assumed to be statistically significant. *: 0.01 < P < 0.05; **: 0.001 < P < 0.01; ***: P < 0.001.
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**Acknowledgments**

This study was supported by the Ministry of Science and Technology of China (2014CB964803), the National Natural Science Foundation of China (31225017 and 91319310), the ‘Strategic Priority Research Program‘ of the Chinese Academy of Sciences (XDA01010403) and the Shanghai Municipal Commission for Science and Technology (12JC1409600 and 13XD1404000).

**Author contributions**

M.Z.: conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing. H.Z., C.Z., E.Z., J.X., W.L. and D.X.: collection and/or assembly of data; Y.S., C.W., H.W. and D.L.: data analysis and interpretation; J.L.: conception and design, data analysis and interpretation, manuscript writing, financial and administrative support, and final approval of manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Zhang, M. et al. The Roles of Testicular C-kit Positive Cells in De novo Morphogenesis of Testis. *Sci. Rep.* **4**, 5936; DOI:10.1038/srep05936 (2014).

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