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Correspondence and requests for materials should be addressed to C.F.L. (chunfang_liu@fudan.edu.cn) or Y.L. (yuanlu@hsh.stn.sh.cn)

* These authors contributed equally to this work.

Activation of the germ-cell potential of human bone marrow-derived cells by a chemical carcinogen

Chunfang Liu^{1*}, Zhan Ma^{1,2*}, Songtao Xu^{3*}, Jun Hou⁴, Yao Hu¹, Yinglu Yu¹, Ruilai Liu¹, Zhihong Chen⁵ & Yuan Lu¹

¹Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, 200040, China,

²Department of Laboratory Medicine, Shanghai Children's Hospital, Shanghai Jiao Tong University, Shanghai 200040, China,

³Department of Thoracic Surgery, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai, 200032, China,

⁴Department of Pathology, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai 200032, China,

⁵Department of Pulmonary Medicine, Research Institute of Respiratory Disease, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai 200032, China.

Embryonic/germ cell traits are common in malignant tumors and are thought to be involved in malignant tumor behaviors. The reasons why tumors show strong embryonic/germline traits (displaced germ cells or gametogenic programming reactivation) are controversial. Here, we show that a chemical carcinogen, 3-methyl-cholanthrene (3-MCA), can trigger the germ-cell potential of human bone marrow-derived cells (hBMDCs). 3-MCA promoted the generation of germ cell-like cells from induced hBMDCs that had undergone malignant transformation, whereas similar results were not observed in the parallel hBMDC culture at the same time point. The malignant transformed hBMDCs spontaneously and more efficiently generated into germ cell-like cells even at the single-cell level. The germ cell-like cells from induced hBMDCs were similar to natural germ cells in many aspects, including morphology, gene expression, proliferation, migration, further development, and teratocarcinoma formation. Therefore, our results demonstrate that a chemical carcinogen can reactivate the germline phenotypes of human somatic tissue-derived cells, which might provide a novel idea to tumor biology and therapy.

It has long been appreciated that tumor and embryonic/germline development share similar traits such as immortalization, invasion, independence, a lack of adhesion, migratory behavior, demethylation, marker expression, and immune evasion^{1–4}. Recently, the germline traits of tumors were reported to play important roles in malignant tumor behaviors⁵. That study led us to reconsider the interesting question of why tumors exhibit extensive embryonic/germline traits. In fact, as recently as 100 years ago, the embryonal-rest tumor hypothesis was proposed; this hypothesis postulated that tumors originated from displaced and activated trophoblasts or even displaced germ cells^{1,2}. However, some researchers proposed that the embryonic/germline traits of tumors should be attributed to the reacquisition of germ-cell development in somatic cells and that this programmatic acquisition is a driving force in tumorigenesis^{3,4,6}. These two concepts are always in dispute because both are supported by strong evidence^{7,8}. Teratomas/teratocarcinomas have been accepted as key pieces of evidence to support the hypothesis of activated trophoblasts or displaced germ cells because these tumors represented embryogenic mimicking and could arise from normal germ cells^{7–11}.

In mammals, the germ-cell lineage is specific and physically separates from the somatic lineages during early embryogenesis¹². Surprisingly, cells derived from mouse bone marrow can be a source of germ cells from which to generate oocytes in adult mice upon entry into the gonads¹³, although it is hard to be reproduced¹⁴. Additionally, germ-cell lineage cells can reportedly be generated from normal somatic tissue-derived cells in specific culture conditions^{15–19}; abnormal somatic tissue-derived cells^{20–23} and induced by transcription factor²⁴, the findings that offer some support to the gametogenic reactivation concept of tumors^{3,4,6}.

Compared with that of normal somatic tissue-derived cells, malignant somatic tissue-derived cells far more efficiently form germ cell-like cells^{15–17,23,24}. Therefore, in the current study, we sought to address whether cancer conditions might have an activating effect on the germ-cell potential of somatic tissue-derived cells. These efforts enabled us to trigger the germ-cell potential of human bone marrow-derived cells (hBMDCs) with a chemical



carcinogen, 3-methyl-cholanthrene (3-MCA), to generate germ cell-like cells, which are similar to human germ cells with regard to their differentiation potential in vitro and teratocarcinoma formation in vivo.

Methods

This study was approved by the Medical Ethical Committee of Huashan Hospital, Fudan University, under permit number MEC-HS (Hu) 2011-362. The ethics committee/institutional review board included Hejian Zou, Yong Gu, Yingyuan Zhang, Chuanzhen Lu, Weihu Fan, Dayou Wang, Jianhua Zhang, Zhongrui Lu, and Quanning Ni. All patients signed informed consent for the collection and use of their bone marrow tissues for this study. All animal experiments were conducted in strict accordance with the National Institutes of Health Guide for the Care.

Isolation and treatment of cells. Leg bones were obtained from injured patients. Human whole bone marrow was collected as previously described²⁰ and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen,) with 10% fetal bovine serum (FBS; PAA Laboratory). One week later, non-adherent cells were discarded and adherent cells were retained. The plastic-adherent cells were used as hBMDCs in this study. After the cultures reached confluence, the primary hBMDCs were subcultured and divided into three groups. One group was collected for DNA and RNA extraction when the secondary hBMDCs reached confluence. One group was induced with 1 μ g/ml of 3-MCA (Sigma) for one week, according to our previous experiments²⁵, after which the induced cells were routinely cultured. The other group underwent routine culture alone as a parallel control.

Culture and differentiation of germ cell-like cells. In accordance with our previous study, the MCA-induced hBMDCs were continuously cultured for 2–6 weeks without subculturing to promote further germ cell-like cell development. Germ cell-like cells appeared spontaneously in the induced hBMDCs and then underwent proliferation, migration, further differentiation into larger germ cell-like cells, and embryo-like structure formation in routine medium. The cultures were observed via microscopy. In order to observe the formation of embryo-like structures from germ cell-like cells, approximately 1000 suspending cells were plated in a semisolid medium with 2.7% methyl cellulose (Sigma), DMEM, and 10% FBS.

Single cell cloning. Cells were diluted and plated at 0.5 cells per well in 96-well plates, and the wells containing single cells were selected after an 8-h incubation at 37°C. Next, the cells were further cultured, and their proliferative and germ cell-like cell formative abilities were observed.

Alkaline Phosphatase Staining. Cultures were incubated with alkaline phosphatase (AP) staining solution (Vector Laboratories) in the dark for 45 min at room temperature after washed twice with a Tris-HCl (pH = 8.2) buffer solution.

RNA Isolation and RT-PCR. Total RNA from different cultures was extracted with Trizol reagent (Invitrogen) and then subjected to reverse transcription with a reverse transcription kit (Invitrogen), according to the manufacturer's instructions. Seminoma cells were used as positive controls. The genes were detected by PCR or qRT-PCR, using cDNA as the template. All genes, primer sequences and product sizes are listed in Supplemental Table S1.

Immunocytochemistry. Cells from different culture time points were grown on or adhered to coverslips. The cells were subsequently fixed with 4% paraformaldehyde and blocked in 1 \times PBS with 5% BSA and 0.05% Triton-X-100 and were subsequently incubated overnight at 4°C with various primary antibodies, including anti-Oct4 (1 : 200; rabbit origin; AbCam), anti-Sox2 (1 : 200; rabbit; AbCam), anti-c-Kit (1 : 80; rabbit; AbCam), anti-Vasa (1 : 200; rabbit; AbCam), anti-DAZL (1 : 150; mouse; AbCam), anti-Nanos3 (1 : 200; rabbit; AbCam), anti-GDF9 (1 : 200; rabbit; AbCam), or SCP3 (1 : 400; rabbit; AbCam). Next, the cells were incubated with Cy3 fluorescent dye-conjugated secondary antibodies (Jackson). The cell nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Invitrogen).

Bisulfite sequencing analysis. Bisulfite treatment of genomic DNAs was performed with the Methylation-Gold Kit™ (Zymo). Nested PCR amplifications were carried out (Supplemental Table S1). The products were sequenced.

Xenograft tumor formation and analysis. Suspended induced cells (mainly containing germ cell-like cells and embryonic-like structures; approximately 1 \times 10⁵ cells/mouse) were injected into five 4-week-old immunodeficient mice (female BALB/c-nude mice) and the mice were sacrificed at 8 weeks. The untreated, routinely cultured cells and primary hBMDCs were also injected into five 4-week-old immunodeficient mice respectively as controls (approximately 1 \times 10⁶ cells per mouse). No tumors were observed in the control mice within their lifespans. The tumors were excised and fixed in 10% neutral-buffered formalin for 24 hours and were subsequently embedded in paraffin wax for further analysis. Tumor sections were routinely stained with hematoxylin and eosin (H&E) or were stained with antibodies against Oct4 (AbCam), Vasa (AbCam), DAZL (AbCam), SCP3 (AbCam), vimentin (Vim; 7 μ g/ml; Chemicon), α -smooth muscle actin (α -SMA; 5 μ g/ml; Chemicon), neurofilament (NF; 1 : 100; Sigma), cytokeratin 18 (CK18; 1 : 200;

AbCam) or a-fetoprotein (AFP; 1 : 200; R&D). The SP kit (Zymed) was used for further immunodetection. Hematoxylin staining was used to show nuclear details.

Induced hBMDCs at day 3 after re-plating (mainly differentiated cells, approximately 5 \times 10⁵ cells/mouse) and at day 20 after re-plating (mainly containing differentiated cells and germ cell-like cells) were injected separately into five 4-week-old immunodeficient mice (female BALB/c-nude mice) to investigate whether germ cell-like cells played a role in tumor formation.

Hormone measurements. Culture media were collected at different time points and measured quantitatively by the Department of Clinical Laboratory Medicine, Huashan Hospital, China to determine the levels of estradiol (E2) and chorionic gonadotropin (CG). Each sample was measured in triplicate, and the means were used in all subsequent analyses.

Statistical analyses. The t-test and SPSS 10 software were used to analyze the data to determine whether there were significant differences between the groups. A P-value < 0.05 was considered significant.

Results

Activation of the germ cell-potential of hBMDCs with a carcinogen. Human BMDCs were obtained from the legs of two volunteers with surgical trauma and were isolated according to plastic adherence. The primary hBMDCs were divided into three groups. Based on our previous experiments²⁵, one group was treated with 3-methyl-cholanthrene (3-MCA) for one week. As a parallel control, the second group underwent routine culture alone. RNA was extracted from the third group for use as primary controls. At three months after 3-MCA treatment completion, germ cell-like cells were observed in the 3-MCA-induced hBMDCs, but not in the parallel control hBMDCs (Figure 1a). From a starting density of 10⁵ hBMDCs, approximately 5000 germ cell-like cell clusters were observed in the induced group (Figure 1b). An immunocytochemistry analysis showed that the germ cell-like cells were positive for germline-specific markers, including Octamer-binding transcription factor 4 (Oct4), Nanos3, c-Kit, Deleted in Azoospermia-like (DAZL) and/or Vasa (Figure 1c). To further address the appearance of the germ cell-like cells, RT-PCR was performed for a series of genes related to germ-cell formation, including *Oct4*, *Stellar*, *Sox2*, *Nanog*, *Ifitm3*, *Nanos3*, *c-Kit*, *DAZL* and *Vasa*. The RT-PCR results showed that these genes were detected in the 3-MCA-induced hBMDCs, but were almost undetectable in the primary hBMDCs and parallel control hBMDCs (Figure 1d). The induced hBMDCs exhibited morphologic changes, rapid growth, anchorage independence, losses of contact inhibition and polarity (no shown), and karyotypic instability (Figure 1e), indicating that these cells had undergone malignant transformation. The findings suggested that 3-MCA, a carcinogen, affected the germ-cell potential activation in the hBMDCs.

Germ cell formation ability at the single-cell level. A single clone analysis was performed to further address the reactivation of germ cell potential in the induced hBMDCs. After replating, the single induced hBMDCs showed a “fibroblast-like” morphology (Figure 2a). At day 7, small round cells with a high nucleus-to-cytoplasm ratio, thus resembling primordial germ cells (PGC), appeared in the cultures (Figure 2a). As the culture time was extended, the germ cell-like cells proliferated and formed cell aggregates (Figure 2a). A subpopulation (approximately 10%) of the germ cell-like cells entered mitotic arrested, gradually increased in diameter and then gave rise to oocyte-like and embryo-like cells (Figure 2a), suggesting an ability to undergo further germ-cell development. Germ cell specification can be initially recognized via staining for tissue non-specific alkaline phosphatase (TNAP), Oct4, and Sox2 during early embryonic development. DAZL is known to be a specific germ cell marker and is required for PGC development and maturation. Some of the round-shaped cells were positive for TNAP, Oct4, Sox, and/or DAZL (Figures 2a and 2b) and were thus consistent with germ cells. However, the fibroblast-like cells were negative for TNAP, Oct4 and DAZL (Figures 2a and 2b) and therefore most likely represented somatic cells. Next, quantitative RT-PCR was performed to assess germ cell formation with respect to the

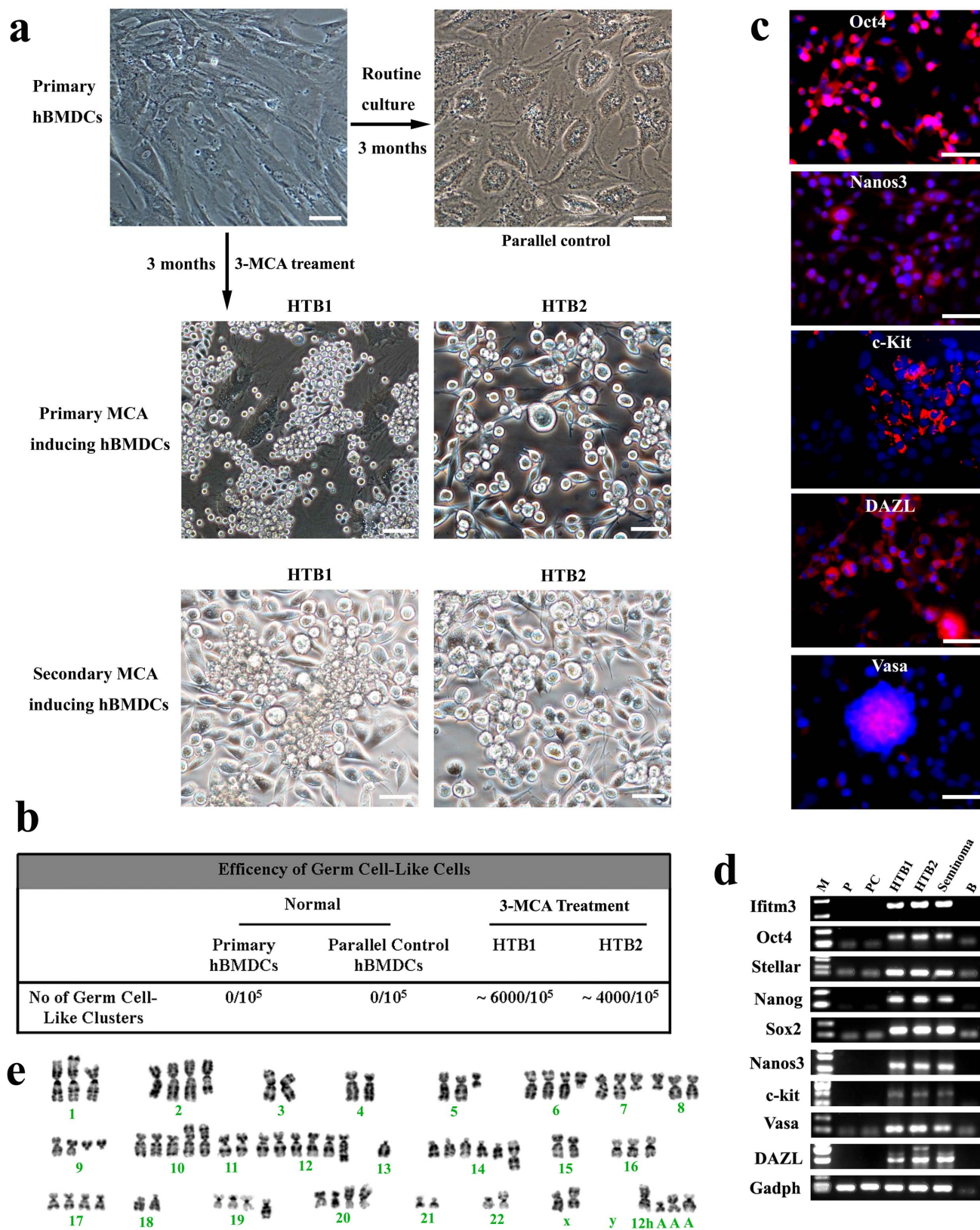
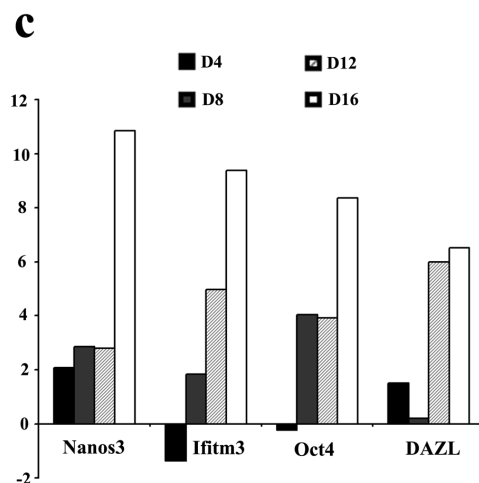
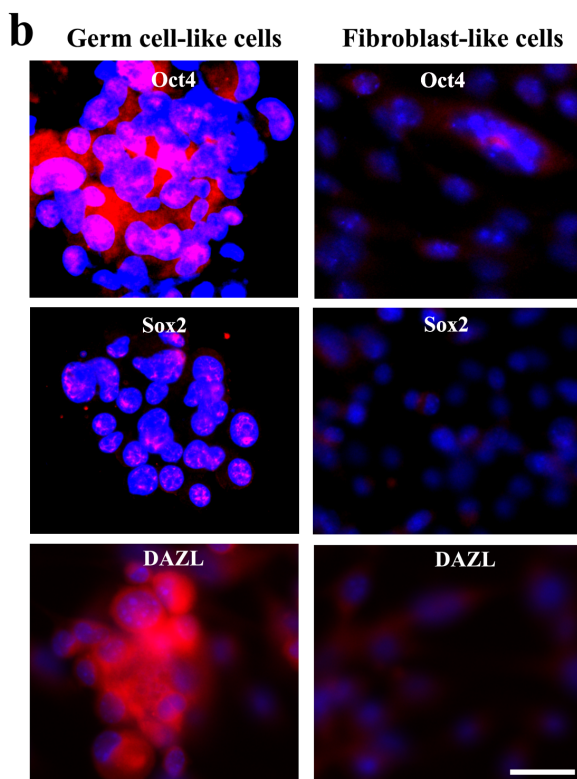
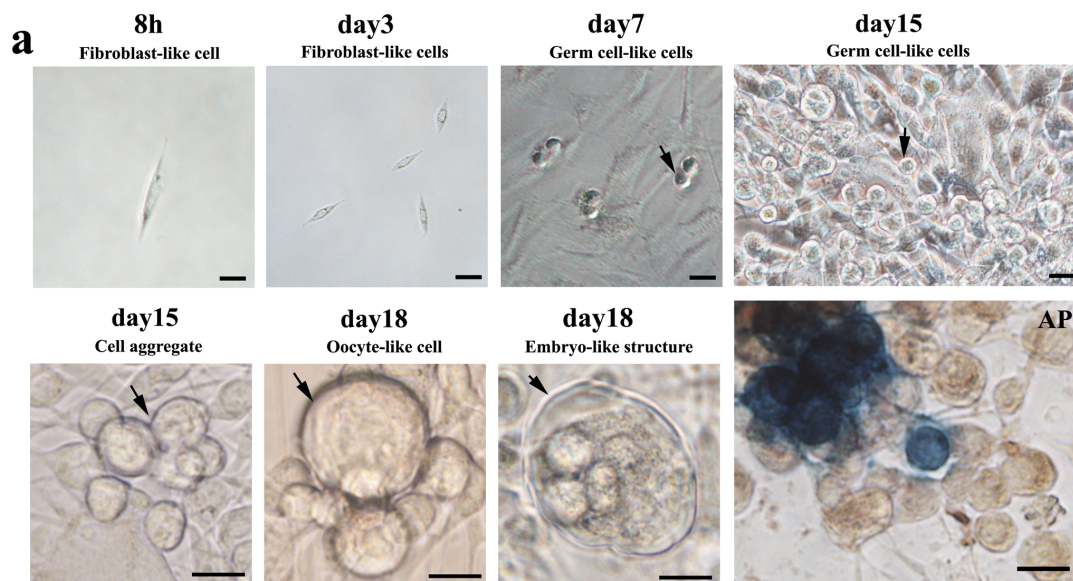


Figure 1 | hBMDC germ cell-potential was activated by a carcinogen. (a) The appearance of germ cell-like cells in 3-MCA-induced hBMDCs. (b) Germ cell-like cell formation efficiency. (c) Germ cell-like cells expressed germ cell markers, including Oct4, Nanos3, c-Kit, DAZL and Vasa (red). Cell nuclei were stained with DAPI (blue). (d) The RT-PCR results showed that germ cell-related markers were detected in induced hBMDCs. Primary hBMDCs (P), parallel control hBMDCs (PC) and induced hBMDCs (HTB1, HTB2) were analyzed. Seminoma cells were used as a positive control. (e) The karyotypes of the 3-MCA-induced hBMDCs (HTB2) were abnormal. Scale bars: 20 μ m in (a); 40 μ m in (c).



d

	Ability of Germ Cell Formation at Single Cell Level within One Month			
	Normal		3-MCA treatment	
	Primary hBMDCs	Parallel Control hBMDCs	HTB1	HTB2
Clone formation	64% (32/50)	56% (28/50)	71.4% (25/35)	75% (30/40)
Germ cell formation	0% (0/50)	0% (0/50)	62.9% (22/35)	65% (26/40)

Figure 2 | Generation of germ cell-like cells from a single transformed hBMDC. (a) Generation of germ cell-like cells from a single induced hBMDC with a differentiated morphology. Germ cell-like cells, oocyte-like cells and embryo-like structures could be derived from a single induced hBMDC. Germ cell-like cells were positive for AP. (b) Germ cell-like cells were positive for Oct4, Sox2 and DAZL staining, while fibroblast-like cells were negative. (c) Quantitative RT-PCR analysis indicated the expression of germ cell formation-specific genes at different culture time points after single-cell culture. (d) Normal hBMDCs and 3-MCA-induced hBMDCs had different abilities with regard to germ cell-like cell formation at a single-cell level. Scale bars: 10 μm in (a); 25 μm in (b).

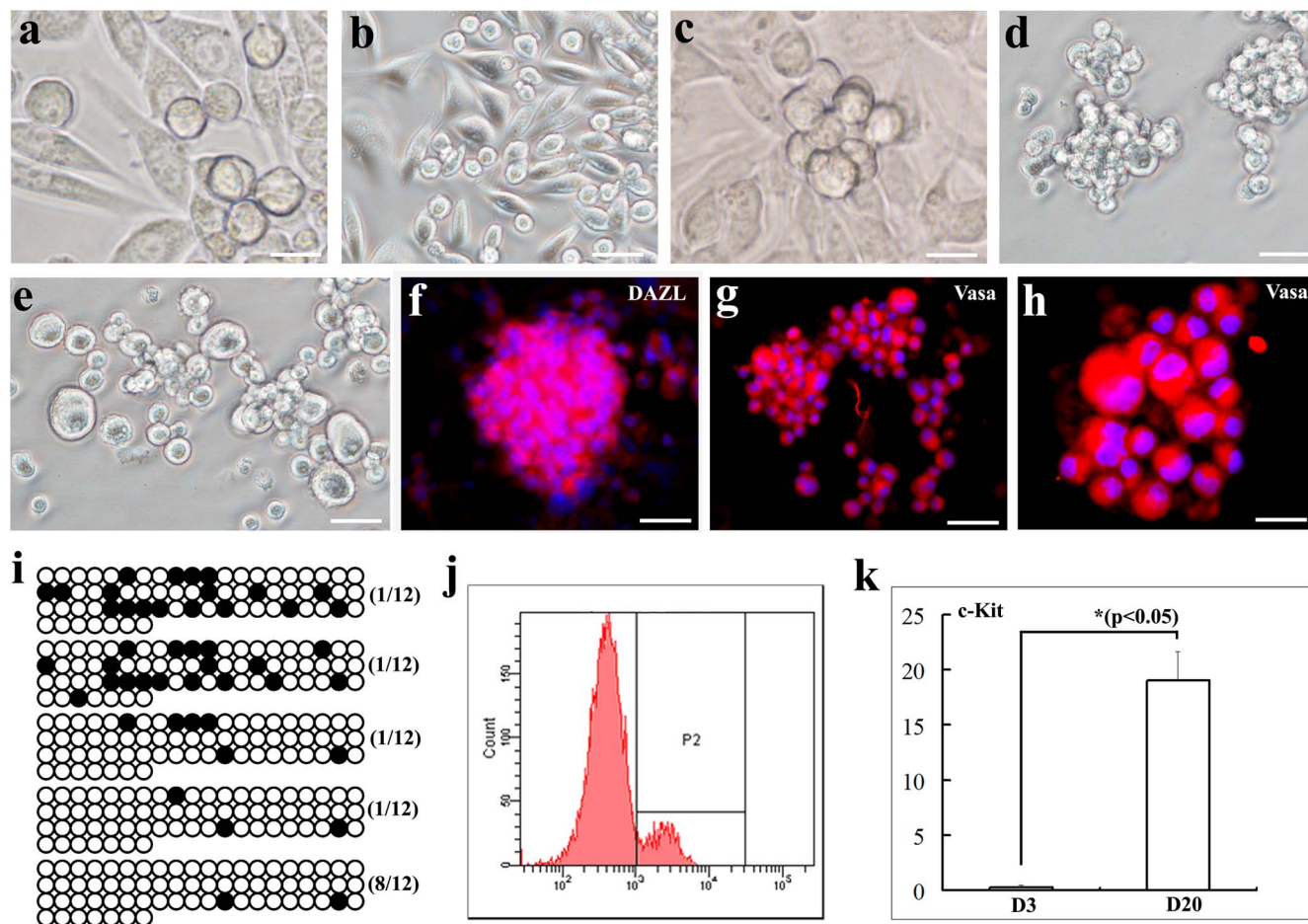


Figure 3 | Characteristics of PGC-like cells in cultures. (a) Early germ cells. (b) The numbers of germ cell-like cells gradually increased. (c) Cell aggregate formation. (d) Appearance of suspended germ cell-like cells. (e) Germ cell-like cells increased in size. (f) DAZL protein-positive cell aggregates. (g) Vasa-positive suspended cell aggregates. (h) Vasa-positive suspended cells with losses of cell-cell contact. (i) Sodium bisulfite sequencing showed that H19 DMR1 hypomethylation appeared in induced hBMDCs. The blank circle represents methylated CpGs, and the white circle represents unmethylated CpGs. (j) Flow cytometric analysis exhibited the ratio of c-Kit-positive cells at day 20 after replating. (k) Flow cytometric analysis showed that the ratio of c-Kit-positive cells increased gradually with the culture time length. Scale bars: 20 μm in (b, d, e, h), 10 μm in (a, c), 40 μm in (f, g).

genes *Ifitm3*, *Oct4*, *Nanos3*, and *DAZL*. As expected, the mRNA levels of these genes generally increased along with the appearance and proliferation of the putative germ cell-like cells (Figure 2c). More than 70% of the single fibroblast-like cells could proliferate and give rise to clones, and germ cell-like cells appeared in most of the single-cell clones within a month (Figure 2d). No germ cell-like cells appeared in the primary hBMDCs or the parallel control hBMDCs within one month at the single-cell level (Figure 2d). The results indicated that germ cell-like cells could develop from a single malignant transformed somatic cell. To further confirm germ cell potential activation, we next investigated the characteristics of the putative germ cells and their derivatives.

Characteristics of primordial germ cell (PGC)-like cells. PGC specification is the first and perhaps most critical event in germ cell development²⁶. Therefore, we should provide more evidence to address the appearances of PGCs, including morphology, proliferation, migration and imprint erasure. The PGC-like cells were round with a high nucleus-to-cytoplasm ratio, and most ranged from 8–12 μm in diameter (Figure 3a). The PGC-like cells proliferated gradually (Figure 3b), formed cell aggregates (Figure 3c) and further develop (Figure 3e), in which most of cells were positive for DAZL at the early (Figure 3f). Flow cytometric analysis showed that the percentages of c-Kit⁺ cells varied from approximately $0.3 \pm 0.2\%$ to $19 \pm 2.64\%$ (Figures 3j and 3k) of the total cell population at

days 3 and 20, respectively, after replating ($p < 0.05$), indicating that the germ cell-like cells can proliferate. With an extended culture time, the individual round cells and cell aggregates began to detach from the plates to float in the medium (Figure 3d), indicating their ability to migrate. The suspended cells generally exhibited reduced cell-cell contact and most exhibited positive Vasa staining (Figures 3g and 3h), consistent with the characteristic of post-migratory germ cells^{27,28}.

During embryonic development, imprint erasure is an epigenetic modification unique to migrating PGCs^{18,28}. *H19* is usually used to assess the epigenetic imprints established during fetal development and gametogenesis^{18,28}. In this study, the H19 DMR1 methylation status was analyzed via sodium bisulfite sequencing to validate the appearance of imprint erasure in induced hBMDCs at day 20 after replating. The methylation analysis results indicated the existence of a germ cell-like subpopulation in which 97% of the CpG sites in *H19 DMR1* were unmethylated (Figure 3i). Above all, the results indicated that PGC-like cells can undergo proliferation, migration and imprint erasure.

Characteristics of female germ cell-like cells. In mammals, oocyte development requires ovarian support, which is mediated by estrogen production^{18,28}. Oocytes cannot grow larger than 25 μm in diameter if estrogen biosynthesis is absent^{18,28}. Structures resembling pre-follicles were observed in the cultures (Figures 4a and 4b), in

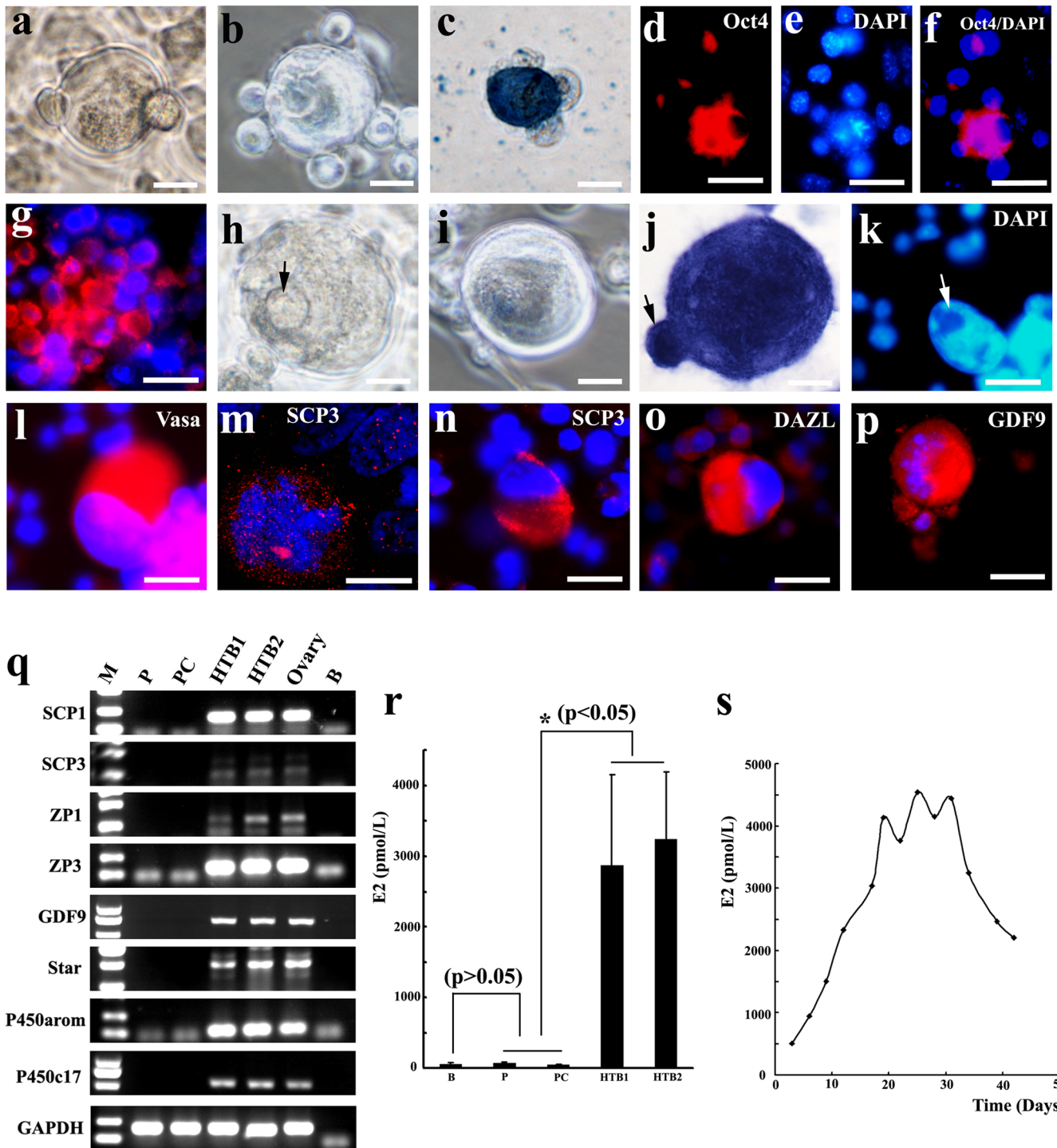


Figure 4 | Appearance of female germ cell-like cells in cultures. (a–b) Phase contrast images of pre-follicle-like structures. (c) A pre-follicle-like structure was stained with AP. The germ cell-like cell was positive, while the surrounding cells were negative. (d–f) Oct4 was detected in a follicle-like structure in which the germ cell-like cell was Oct4-positive. (g) P450 arom was detected in the cultures. (h) Phase contrast images of an oocyte-like cell with a GV-like structure (arrow). (i) An oocyte-like cell with a zona pellucida-like membrane (arrow). (j) Giemsa staining showed an oocyte-like cell with a polar body-like structure (arrow). (k, l) Vasa-positive oocyte-like cells. (k) DAPI staining showed a GV-like structure (arrow). (m–p) SCP3, DAZL and GDF9 detection in oocyte-like cells. (q) RT-PCR analysis of follicle and oocyte-related genes. (r) The estradiol levels increased in induced hBMDC cultures. (s) The estradiol levels increased in induced hBMDC cultures as the culture time increased. Scale bars: 10 μ m in (a–c, h–j), 20 μ m in (d–g, k–p).

which the bigger cells were positive for TNAP (Figure 4c) and Oct4 protein (Figures 4d–4f), while the surrounding cells were negative for TNAP (Figure 4c) and Oct4 protein (Figures 4d–4f). Compared with normal hBMDC cultures, the induced hBMDCs could produce estradiol (Figures 4r and 4s). P450 arom, a key protein in estrogen

biosynthesis, was expressed in some of the malignant transformed hBMDCs (Figure 4g), thus further supporting the estrogen levels.

The oocyte-like cells were round or ovoid, and most grew to 35–50 μ m in diameter (Figures 4h–4j). Zona pellucida-like structures (Figure 4i) were visible in only approximately 2% of the oocyte-like

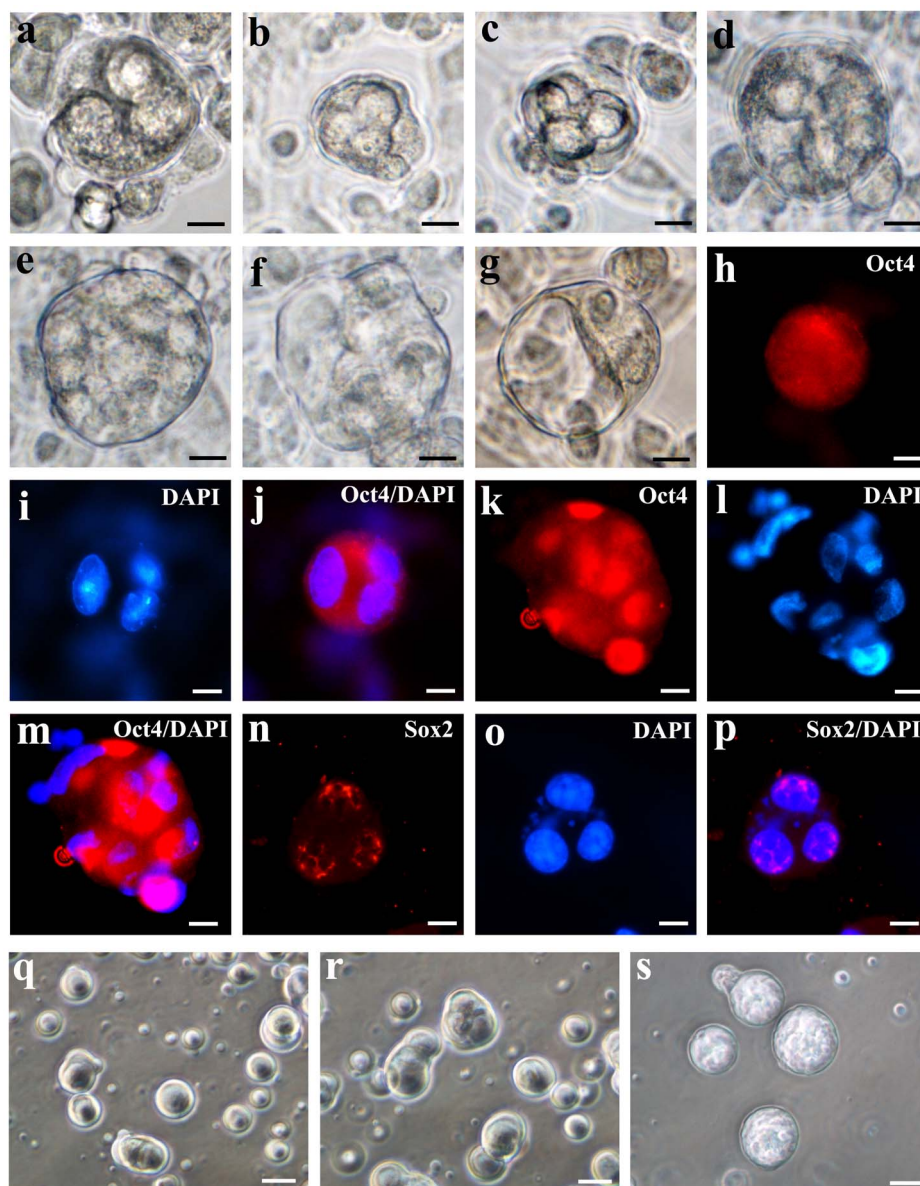


Figure 5 | Formation of embryo-like structures in culture. (a–g) Appearance of embryo-like structures, from cleavage stage-like embryos to blastocyst-like structures, in culture. (a) Three cell stage. (b–d) Early embryo-like structures at the cleavage stage. (e) Morula-like embryo. (f–g) Blastocyst-like structures with compacted cells. (h–j) Oct4 expression in a three cell-stage-like embryo. (k–m) Oct4 expression in an early embryo-like structure. (n–p) Sox2 expression in a three cell-stage-like embryo. (q–s) The derivation of embryo-like structures from germ cell-like cells in semi-solid medium. (s) Formation of embryo-like structures with a high compacted morphology. Scale bars: 10 μ m.

cells. Immunohistochemical staining indicated that Vasa (Figure 4l), DAZL (Figure 4o), and growth differentiation factor (GDF9) (Figure 4p) proteins were detectable in the oocyte-like cells, further suggesting their similarities to natural oocytes with regard to protein expression. GDF9 expression also correlated with the formation of pre-follicle-like structures; this growth factor is produced by oocytes and is essential for normal folliculogenesis^{18,28}.

Synaptonemal complex proteins 3 (SCP3) is a component of the synaptonemal complex, which is essential for meiotic entry. Germ cells of approximately 25 μ m in size exhibited nuclear SCP3 expression (Figure 4m). In larger germ cell-like cells, SCP3 was localized mainly within the cytoplasm (Figure 4n), suggesting that these cells were at a more advanced meiotic stage²⁷. Germinal vesicle (GV)-like structures (Figures 4h and 4k) were observed in approximately 20% of the oocyte-like cells. Polar body (PB)-like structures were observed in approximately 1% of the oocyte-like cells (Figure 4j), indicating completion of the first phase of meiosis.

RT-PCR results showed that genes related to follicle structures, estrogen biosynthesis and oocyte cells were detected in induced hBMDCs at day 20 after re-plating (Figure 4q), including *GDF9*, *DAZL*, *Vasa*, *zona pellucida* (*ZP*, including *ZP1* and *ZP3*), meiotic markers (*synaptonemal complex protein*, *SCP1* and *SCP3*) and key genes involved in estrogen biosynthesis (*steroidogenic acute regulatory protein*, *StAR*, *P450 17-hydroxylase-17/20 lyase*, *CYP17*, and *P450 arom*).

Formation of early embryo-like structures. Early embryo-like structures representing different developmental stages were observed in the cultures (Figures 5a–5g). Most of the structures varied from 20–50 μ m in size (Figures 5a–5g). A defined membrane resembling a zona pellucida was not observed around the embryo-like structures. Trophectoderm layer-like structures were also not observed, and HCG production was undetectable in the cultures (not shown). To further evaluate their similarities to

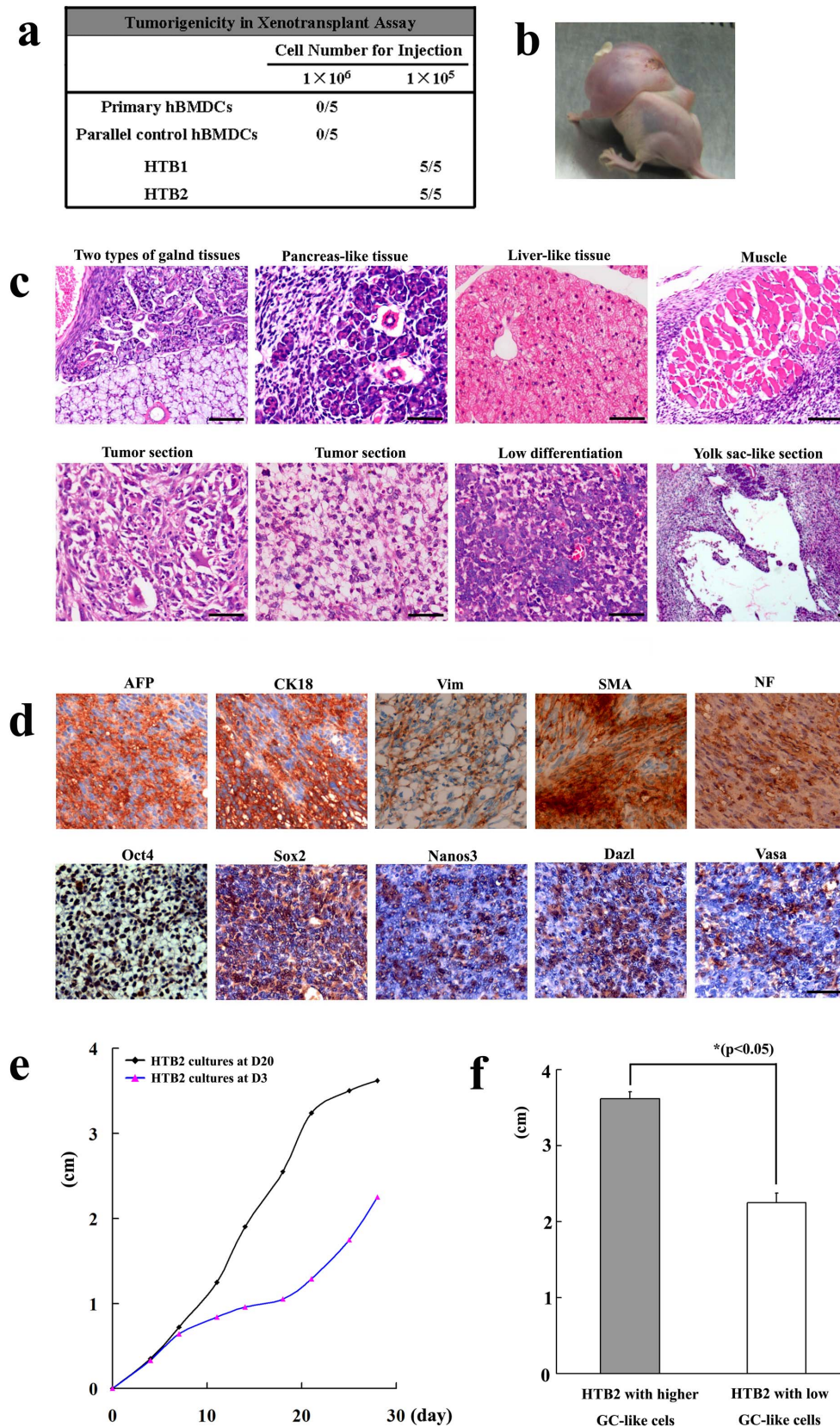


Figure 6 | Teratocarcinoma formation. (a) Tumorigenicity of induced HTB1, HTB2 and control cells. (b) Tumor from induced HTB2. (c) Teratocarcinoma with well differentiated tissue, tumor tissue, germinal tumor, yolk sac tumor, and embryonic tumor areas. (d) Immunohistochemical staining showed the expression of proteins specific for somatic differentiation (CK8, AFP, Vim, SMA, and NF) and germ-cell differentiation (Oct4, Sox2, DAZL, Nanos3 and Vasa) in the tumors. (e) Tumor growth curves derived from induced hBMDCs at day 20 and at day 3 after re-plating. (f) Tumors derived from the induced hBMDCs at day 20 and at day 3 after re-plating were 3.62 ± 0.09 cm ($n=5$) and 2.25 ± 1.13 cm ($n=5$) in diameter, respectively ($p < 0.05$). Scale bars: 25 μ m.



natural embryos, Oct4 (Figures 5h–5m) and Sox2 (Figures 5n–5p) proteins were detected in the embryo-like structures. Consistent with other reports²⁷ of mammalian embryos, the Oct4 protein was expressed and distributed in the cytoplasm in the very early embryo-like structures, after which it began to localize in the nucleus (Figures 5h–5m). The embryo-like structures derived from germ cell-like cells in semi-solid medium (Figures 5q–5s). These results indicated that blastocyst-like structures could be derived from induced hBMDCs.

Teratocarcinoma formation. Reportedly, germ cells can generate germinal tumors or teratomas/teratocarcinomas *in vivo*^{9–11}. To investigate the similarities to natural germ cells with regard to tumor formation, the suspended cells, which represented germ cell-like cells and their derivatives, were injected into BALB/c-nude mice. Primary hBMDCs and parallel-cultured hBMDCs were used as controls. Tumors were observed at the injection sites of all nude mice injected with induced hBMDCs within a week; however, no tumors were observed in the mice injected with primary hBMDCs or parallel-cultured hBMDCs during their life spans (Figure 6a). The tumors derived from induced hBMDCs grew very rapidly and exceeded 3 cm in diameter within eight weeks (Figure 6b). Histological and immunohistochemical analysis (Figures 6c and 6d) showed that these tumors exhibited traits associated with teratocarcinomas, which mimic embryonic development. Teratocarcinoma formation from the germ cell-like cells further supports the activation of germ-cell potential in induced hBMDCs.

Contributions to tumor growth. Subsequently, we further addressed whether the germ cell-like cells played a role in the malignant behaviors observed in the induced hBMDCs. Induced hBMDCs (HTB2) at day 20 after re-plating (mainly containing differentiated cells and germ cell-like cells) and at day 3 after re-plating (mainly differentiated cells) were injected separately into five BALB/c-nude mice. Tumors derived from the former group grew rapidly than those derived from the latter (Figures 6e and 6f), suggesting that the germ cell-like cells might benefit tumor growth. All tumors derived from the two groups exhibited characteristics of teratocarcinomas. No macroscopic visible metastases were observed in distant tissues.

Discussion

Our results reveal that the chemical carcinogen 3-MCA might be an important factor that triggers the germline potential of somatic cells. The germ cell-like cells from induced hBMDCs were similar to natural germ cells with respect to morphology, gene expression, proliferation, migration, further development and teratocarcinoma formation. Additionally, the germ cell-like cells is a neoplastic counterpart of normal germ cells and might contribute to tumor growth. Future studies will address whether germ-cell formation potential of somatic tissue-derived cells can be reactivated in other cancer condition. In contrast to germ cell potential of normal somatic tissue-derived cells in specific culture conditions^{15–18}, malignant hBMDCs could spontaneously form germ cell-like cells at a higher efficiency in routine culture. Therefore, it is possible that the reactivation of germ-cell potential *in vivo* might be closely linked to the pathological state of carcinogenesis, which might involve a malignant tumor formation mechanism.

The origin of germ cell-like cells is an important question that presents remaining issues. One possibility is that some PGCs are 'lost' during migration and reside in the bone marrow, where their germ-cell potential is retained. Alternatively, somatic cells might reserve an intrinsic germ-cell formation potential that can be reactivated under specific conditions. As we know, germ cells can generate teratoma/teratocarcinomas *in vivo* and further develop into more mature germ cells *in vitro*²⁷. However, neither teratomas/teratocar-

cinomas nor germ cell-like cells formed in the parallel-cultured hBMDCs. Therefore, our results indicate that germ cell-like cell formation might not be a natural trait of hBMDCs⁷ but rather a reacquired trait after chemical carcinogen induction. The similar activation of soma-to-germline transformation has been demonstrated in mutant *C. elegans*, in which it contributed to increased longevity²⁹. The activation of soma-to-germline transformation in carcinogenesis would provide support to the gametogenesis-related tumor theories^{1–4,6} and would likely facilitate our understanding of the origins and etiologies of germinoma, teratomas and teratocarcinomas. That is, somatic tissue cells might be a candidate for the origins of germinoma, teratomas and teratocarcinomas, especially outside the genital ridge. Cancers vary greatly and are complicated; therefore, we propose that both soma-to-germline transformation and germ cell residence might cause cancer. Further study is needed to address how soma-to-germline transformation is activated in carcinogenesis.

Tumors' malignant behaviors involve in many independent events, thus it is almost impossible that these behaviors just attribute to cells at a special stage. However, the reactivation of germline/embryonic traits in tumors can almost link all the malignant events of tumors together. Therefore, our findings provide a novel idea to tumor biology and therapy.

Germ cell-like cells in cancer would confer upon tumors these traits, such as immortalization, invasion, migration, and immune evasion, among others, that are related to the tumors' central malignant traits. Our results also indicated that germ cell-like cells might play roles in tumor growth. Therefore, killing germ cell-like cells and inhibiting the reactivation of the germ-cell fate in somatic cells might be potential ways to prevent malignant tumor behaviors. However, future studies will reveal whether germ cell-like cells exist in natural human tumors and play key roles in malignant tumor behaviors.

Germ cell-like cell formation in malignant hBMDCs is similar but not identical to natural human gametogenesis; rather, it seems to mimic gametogenesis, and therefore some of these processes might be not correctly orchestrated. For example, oocyte-like cells could not fully mature, and their embryonic derivatives lacked trophoderm-like structures. Both male and female ES cells can demonstrably differentiate into female germ cells²⁷. Therefore, it is possible that both male and female somatic cells might have the potential to generate female germ cell-like cells during the course of carcinogenesis. Future experiments will reveal whether the reactivation of germ-cell potential indeed appears *in vivo* and causes tumor formation in cancer.

1. Bignold, L. P., Coghlan, B. L. & Jersmann, H. P. Hansemann, Boveri, chromosomes and the gametogenesis-related theories of tumours. *Cell Biol. Int.* **30**, 640–644 (2006).
2. Brewer, B. G., Mitchell, R. A., Harandi, A. & Eaton, J. W. Embryonic vaccines against cancer: An early history. *Exp. Mol. Pathol.* **86**, 192–197 (2009).
3. Old, L. J. Cancer/testis (CT) antigens - a new link between gametogenesis and cancer. *Cancer Immun.* **1**, 1 (2001).
4. Old, A. J., Caballero, O. L., Jungbluth, A., Chen, Y. T. & Old, L. J. Cancer/testis antigens, gametogenesis and cancer. *Nat. Rev. Cancer* **5**, 615–625 (2005).
5. Janic, A., Mendizabal, L., Llamazares, S., Rossell, D. & Gonzalez, C. Ectopic expression of germline genes drives malignant brain tumor growth in *Drosophila*. *Science* **330**, 1824–1827 (2010).
6. Old, L. J. Cancer is a somatic cell pregnancy. *Cancer Immun.* **7**, 19 (2007).
7. Ratajczak, M. Z. *et al.* Epiblast/germ line hypothesis of cancer development revisited: lesson from the presence of Oct-4+ cells in adult tissues. *Stem Cell Rev.* **6**, 307–316 (2010).
8. Visvader, J. E. & Lindeman, G. J. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer* **8**, 755–768 (2008).
9. Stevens, L. C. Origin of testicular teratomas from primordial germ cells in mice. *JNCI* **38**, 549–552 (1967).
10. Stevens, L. C. Animal model of human disease: benign cystic and malignant ovarian teratoma. *Am. J. Pathol.* **85**, 809–813 (1976).
11. Stevens, L. C. Spontaneous and experimentally induced testicular teratomas in mice. *Cell Differ.* **15**, 69–74 (1984).



12. Schöler, H. R. & Wu, G. Oocytes originating from skin? *Nat. Cell Biol.* **8**, 313–314 (2006).
13. Johnson, J. *et al.* Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122**, 303–315 (2005).
14. Eggan, K., Jurga, S., Gosden, R., Min, I. M. & Wagers, A. J. Ovulated oocytes in adult mice derive from non-circulating germ cells. *Nature* **441**, 1109–1114 (2006).
15. Danner, S., Kajahn, J., Geismann, C., Klink, E. & Kruse, C. Derivation of oocyte-like cells from a clonal pancreatic stem cell line. *Mol. Hum. Reprod.* **13**, 11–20 (2007).
16. Dyce, P. W., Wen, L. & Li, J. In vitro germline potential of stem cells derived from fetal porcine skin. *Nat. Cell Biol.* **8**, 384–390 (2006).
17. Dyce, P. W. *et al.* In vitro and in vivo germ line potential of stem cells derived from newborn mouse skin. *PLoS One* **6**, e20339 (2011).
18. Linher, K., Dyce, P. & Li, J. Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells. *PLoS One* **4**, e8263 (2009).
19. Nayernia, K. *et al.* Derivation of male germ cells from bone marrow stem cells. *Lab. Invest.* **86**, 654–663 (2006).
20. Liu, C. *et al.* Generation of pluripotent cancer-initiating cells from transformed bone marrow-derived cells. *Cancer Lett.* **28**, 140–149 (2011).
21. Liu, C. *et al.* Germline traits of human hepatoblastoma cells associated with growth and metastasis. *BBRC.* **437**, 120–126 (2013).
22. Ma, Z. *et al.* Spontaneous generation of germline characteristics in mouse fibrosarcoma cells. *Sci. Rep.* **2**, 743 (2012).
23. Ma, Z. *et al.* Spontaneous germline potential of human hepatic cell line in vitro. *Mol. Hum. Reprod.* **19**, 216–226 (2013).
24. Nakaki, F. *et al.* Induction of mouse germ-cell fate by transcription factors in vitro. *Nature* **501**, 222–226 (2013).
25. Liu, C., Chen, Z., Chen, Z., Zhang, T. & Lu, Y. Multiple tumor types may originate from bone marrow-derived cells. *Neoplasia* **8**, 716–724 (2006).
26. Conti, M. & Giudice, L. From stem cells to germ cells and back again. *Nat. Med.* **14**, 1188–1190 (2008).
27. Hübner, K. *et al.* Derivation of oocytes from mouse embryonic stem cells. *Science* **300**, 1251–1256 (2003).
28. Nicholas, C. R., Chavez, S. L., Baker, V. L. & Reijo Pera, R. A. Instructing an Embryonic Stem Cell-Derived Oocyte Fate: Lessons from Endogenous Oogenesis. *Endocr. Rev.* **30**, 264–283 (2009).
29. Curran, S. P., Wu, X., Riedel, C. G. & Ruvkun, G. A soma-to-germline transformation in long-lived *Caenorhabditis elegans* mutants. *Nature* **459**, 1079–1084 (2009).

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Author contributions

This study was conceived and designed by L.C. and Lu Y., L.C., M.Z., H.Y., L.R. and Y.Y. performed the experiments. X.S. collected the blood sample. Hou J performed the pathologic diagnose. L.C. wrote the main manuscript text. M.Z., L.C., X.S. and C.Z. analysed the data.

Additional information

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