



# Spontaneous generation of germline characteristics in mouse fibrosarcoma cells

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Received  
16 March 2012

Accepted  
19 September 2012

Published  
17 October 2012

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SUBJECT AREAS:  
DIFFERENTIATION  
DEVELOPMENTAL BIOLOGY  
ONCOGENESIS  
DEVELOPMENT

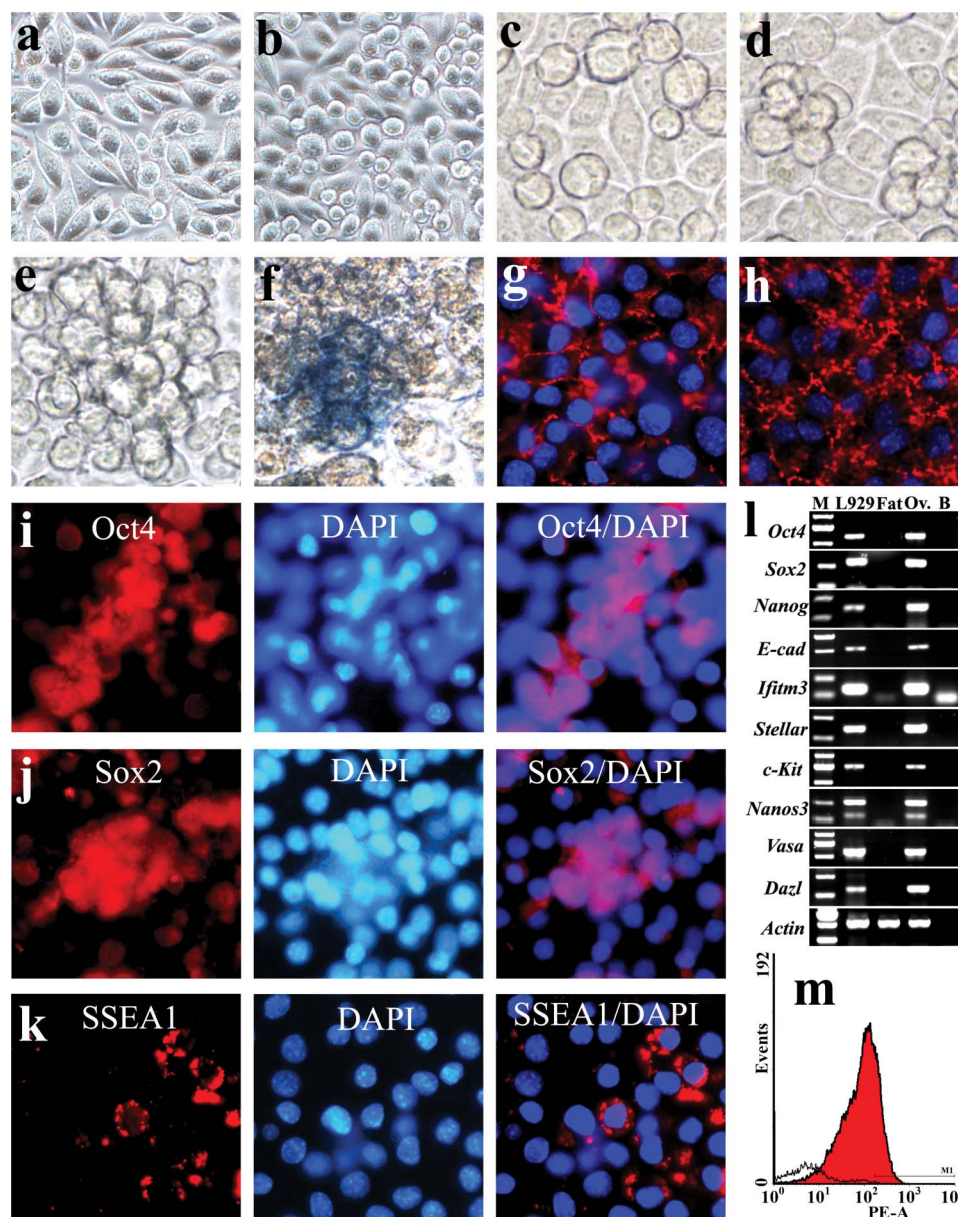
Germline/embryonic-specific genes have been found to be activated in somatic tumors. In this study, we further showed that cells functioning as germline could be present in mouse fibrosarcoma cells (L929 cell line). Early germline-like cells spontaneously appeared in L929 cells and further differentiated into oocyte-like cells. These germline-like cells can, in turn, develop into blastocyst-like structures *in vitro* and cause teratocarcinomas *in vivo*, which is consistent with natural germ cells in function. Generation of germline-like cells from somatic tumors might provide a novel way to understand why somatic cancer cells have strong features of embryonic/germline development. It is thought that the germline traits of tumors are associated with the central characteristics of malignancy, such as immortalization, invasion, migration and immune evasion. Therefore, germline-like cells in tumors might provide potential targets to tumor biology, diagnosis and therapy.

It has been noted for a long time that germ-cell development and tumor formation share important similarities<sup>1–3</sup>. For instance, immortalization, invasion, independence, lack of adhesion, migratory behavior, demethylation, and immune evasion are examples of features and processes shared by cancer cells and cells undergoing germ cell/gamete/trophoblast differentiation<sup>1–3</sup>. Indeed, as early as 100 years ago, based on the similarity of the biological features of trophoblasts and cancer cells, Wright (1910) proposed a germinal cell origin of a pediatric sarcoma, i.e., Willm's tumor (nephroblastoma), and John Beard (1911) postulated that tumors may arise from displaced and activated trophoblasts or even displaced germ cells (GCs)<sup>1–3</sup>. Germ cells can generate gametes (oocytes and spermatocytes), and trophoblastic cells that contribute to the formation of the chorion and the placenta<sup>1</sup>.

Evidence for an association between the processes of germline development and tumor formation comes in two main forms<sup>1,2</sup>. First, germline tumors are known to occur in testicle or ovary tissues, as observed in ovarian and yolk-sac tumors as well as seminomas, teratomas, and teratocarcinomas<sup>1,2,4</sup>. Germline tumors can even occur outside of the genitals, as is the case for mediastinal GC and brain GC tumors<sup>4</sup>. Second, it is well known that germline-related genes, such as the so-called cancer testis (C/T) antigens, are often found to be activated (~40 identified so far) in various tumors (e.g., gastric, lung, liver, renal, and bladder carcinomas as well as melanomas, medulloblastomas, pediatric sarcomas, and germinal tumors<sup>1,3–9</sup>). Intriguingly, germline genes are frequently co-expressed in somatic tumors, therefore Lloyd J. Old proposed that the activation of germline genes in tumors might reflect the activation of the silenced gametogenic programme in somatic cells, and that this programmatic acquisition is one of the driving forces of tumorigenesis<sup>1,3</sup>. We further proposed that the activation of a gametogenic program might reflect the formation of germ cells in somatic tumor cells.

Janic et al. reported that germline traits are necessary for tumor growth and that inactivation of germline genes can have tumor-suppressing effects in *Drosophila*<sup>10</sup>, which indicated that the acquisition of germline/embryonic traits contributes to tumor malignancy<sup>1,3,10–12</sup>. Therefore, it is essential to determine if cells that function as germ cells are present in somatic cancers.

It has reported that germline cells could be generated from somatic cells under special culture condition<sup>13–16</sup>. In our previous study, we found that oocyte-like cells can be generated from bone marrow-derived cells by treatment with the carcinogen 3-methylcholanthrene<sup>17</sup>, suggesting the possibility of germ cell formation in carcinogenesis. However, this result may represent an extreme case because bone marrow-derived cells are known for their



**Figure 1 | Formation and marker expression in PGC-like cells.** (a) Phase contrast images of L929 cells in culture. (b) Small, round cells appeared above the L929 cells at day 7 of culture. (c) Small, round cells in high magnification. (d) Formation of cell aggregates. (e) A bigger cell aggregate. (f) A few germ-like cells positive for AP. Expression of Vim (g) and fibrin (h) in fibroblast-like cells. Oct4 (i), Sox2 (j), and SSEA1 (k) were detected in cultures with germ-like cells. (l) The results of the RT-PCR analysis, showing the genes that were related to germ cell formation expressed in cultures at day 12. The DNA size makers (M) are indicated in the first lane. Normal subcutaneous areolar and adipose tissue (Nt) and ovary (Ov.) was used as a control. (m) Flow cytometry analysis showed that the ratio of SSEA1<sup>+</sup> cells were  $0.5 \pm 0.72\%$  ( $n=3$ ) and  $30.17 \pm 3.98\%$  ( $n=3$ ) of in cultures at day 3 and day 12 respectively ( $P < 0.05$ ). Scale bars = 20  $\mu$ m.

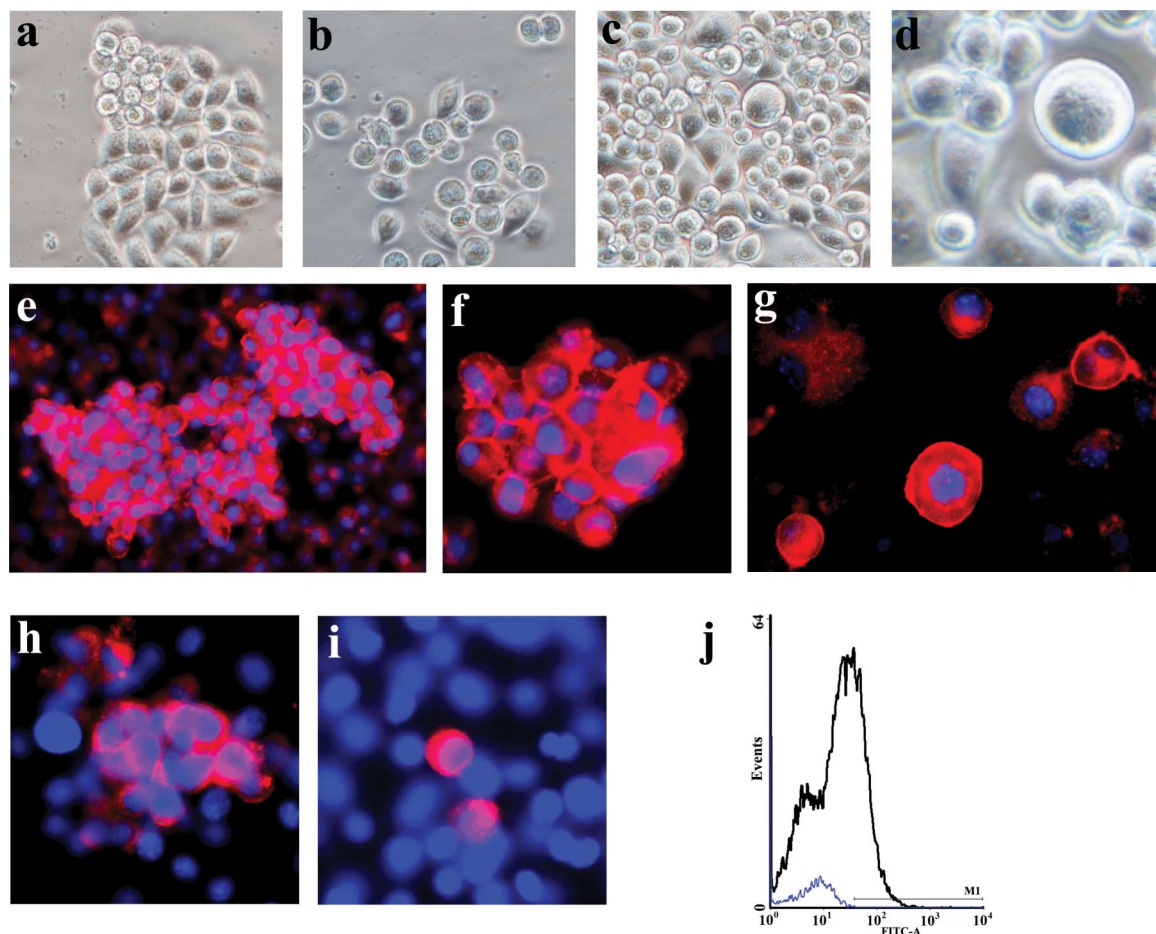
multipotency; they can differentiate into cells of all three germ layers<sup>18–20</sup> even female or male germ cells<sup>21,22</sup> under certain condition. In order to show that the gametogenesis-like phenomenon is common in tumors, we further investigate whether tumor cells derived from mouse adult somatic tissues are able to undergo germline differentiation.

## Results

**Formation of germline-like cells.** The parent of fibrosarcoma cell line L929 is derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse, and they represent thus adult somatic-derived cells. Clone L929 was established (by the capillary technique for single cell isolation) from the 95th subculture

generation of the parent strain. L929 cells were neoplastic and heterogeneous<sup>23,24</sup>. Accordingly, L929 cells representing tumors of adult somatic-derived tissue origin were used as a model to investigate germ cell formation in this study.

The L929 cells exhibited adherent growth and heterogeneous in morphology, including spindle-like, epithelial-like, stellate, and round shape (Figure 1a). The L929 cells expressed Vim (Figure 1g) and Fib (Figure 1h). To facilitate the study of germ cell formation, the cells were continuously cultured for 2–4 weeks without subculture such that they reached full confluence and high density. Approximately 7 days later after re-plate, the adherent differentiating cells proliferated to form a confluent layer of cells, and the small round-shaped cells with a high nucleus-to-cytoplasm ratio, resembling primordial germ cells (PGCs), grew above the confluent layer in



**Figure 2 | Further differentiation of early PGCs.** (a) Suspending cell aggregates. (b) Suspending individual germline-like cells. (c) Appearance of bigger cells. (d) A bigger germline-like cells in high magnification. Expression of *c-kit* protein in a subpopulation of germline-like cells. Immunocytochemistry showed that *DAZL* was detected in germline-like cell aggregates (e), suspending cell aggregates (f), and individual germline-like cells with different diameter (g). Immunocytochemistry showed that *Vasa* were detected in cell aggregates (h) and individual germline-like cells (i). (j) Flow cytometry analysis showed the ratio of *Vasa*<sup>+</sup> cells were in cultures at day 25. Scale bars = 10  $\mu$ m in (a-d), 25  $\mu$ m in (e-i).

culture (Figure 1b, c). The small, round-shaped cells were approximately 5–8  $\mu$ m in diameter at the beginning. After about 10 days of culture, these small, round-shaped cells formed cellular aggregates (Figure 1d, e) that were similar to the clusters formed by PGCs *in vivo*<sup>25</sup>. The shape of the cellular aggregates was different from the granulated colonies of EC/ES cells. The results suggested that germline-like cells might appear in cultures.

**Expression of germline-related genes.** During the course of specification, PGCs express *Oct4*, *Sox2*, *Stellar*, *Ifitm3*, *AP*, *SSEA1*, *Nanos3*, *E-Cadherin* (*E-Cad*), and *c-Kit*<sup>19</sup>. Thus, RT-PCR was performed to detect these genes during the process of germline-like cell formation in L929 cells. The RT-PCR results showed that *Oct4*, *Sox2*, *Nanog*, *Nanos3*, *Stellar*, *Fragilis*, *E Cadherin* and *Blimp1* were detected in the cultures (Figure 1l), indicating that early germline-specific genes are activated in L929 cells. Immunocytochemistry staining for *Oct4* (Figure 1i), *Sox2* (Figure 1j), and *SSEA1* (Figure 1k) indicated that a subpopulation of round-shaped cells in our cultures expressed markers similar to those of PGCs. As expected, germline protein markers were not homogeneously expressed in all tumor cells but expressed only in a relatively small proportion of L929 cells, which is consistent with the expression of CT antigens in tumor tissues<sup>3</sup>. To estimate the ratio of early germline-like cells in cultures, *SSEA1*<sup>+</sup> cells were detected by flow cytometry. The results showed that the ratio of *SSEA1*<sup>+</sup> cells were almost undetectable at day 3 and increased gradually with the extension of

culture time (Figure 1m). The ratio of *SSEA1*<sup>+</sup> cells were  $0.5 \pm 0.72\%$  ( $n=3$ ) and  $30.17 \pm 3.98\%$  ( $n=3$ ) of the total cell population at day 3 and day 12 respectively (Figure 1m). However, AP activity, which is a marker of PGCs, was almost undetected in the putative PGCs (Figure 1f). Twenty independent cultures were used to observe the ability of germline-like cell formation in L929 cells. The similar results were obtained in all the independent experiments. Collectively, these findings suggested that the L929 cells could generate early germline-like cells, which are similar to natural PGCs in morphology and marker expression.

**Further development of early germline-like cells.** At 12 days after re-plate, a subpopulation (approximately 10%) of cell aggregates and individual germ-like cells began to detach from the plate and became motile (Figure 2a, b), suggesting that these cells loss cell-cell contact which might be similar to migratory/postmigratory germline cells<sup>26</sup>. At about day 20, approximately 70% germline-like cells and cell aggregates suspended in media. At day 12, approximately 5% of round-shaped cells increased gradually in diameter (Figure 2c, d), further indicating that the germline-like cells could undergo further development. *DAZL* is known to be expressed in germ cells and is required for the development of PGCs and for their differentiation and maturation<sup>13</sup>. Expression of the RNA helicase enzyme *Vasa* begins in post-migratory PGCs and lasts until the postmeiotic stage of oocytes<sup>26</sup>. Therefore, *Vasa* is useful indicator of the presence of post-migratory PGCs<sup>25,26</sup>. Immunocytochemistry staining showed



that both *DAZL* (Figure 2e–g) and *Vasa* (Figure 2h, i) are expressed in a subpopulation of individual germline-like cells and cell aggregates. The appearance of *Vasa*<sup>+</sup> germline-like cells suggested that we were observing postmigratory PGC-like cells<sup>25,26</sup>. The results of the RT-PCR experiments further showed that *DAZL* and *Vasa* were expressed in the cultures (Figure 1l). Flow cytometry analysis showed that the ratio of *Vasa*<sup>+</sup> cells were approximately  $22.96 \pm 1.45\%$  ( $n=3$ ) of the total cell population at day 20 (Figure 2j).

**Formation of female germline cells.** Upon further culture, round or ovoid-shaped cells were present in cultures (Figure 3a–c), resembling gonocytes or primitive oocytes in morphology. Approximately 98% of the oocyte-like cells were 20–25  $\mu\text{m}$  in diameter (Figure 3a, c). A few oocyte-like cells could reach up to about 40  $\mu\text{m}$  (Figure 3c). A zona pellucida-like membrane was unobvious around the oocyte-like cells. The immunocytochemistry results showed that these round, large cells expressed *DAZL* (Figure 3d–f) and *Vasa* protein (Figure 3g–m), suggesting their identity as germline cells. To further confirm the identity of the bigger germline-like cells, RT-PCR was performed to detect oocyte-specific genes, including *zona pellucida* genes (*ZP1*, *ZP2*, *ZP3*) and *Growth/differentiation factor 9* (*Gdf9*). The results of the RT-PCR showed that *ZP2*, *ZP3*, and *Gdf9* were present in the oocyte-like cells (Figure 3q). However, *ZP1* was undetectable in the larger germline-like cells (Figure 3q).

Follicle-like structures were not observed, and estradiol (E2) was undetectable in cultures (Figure 3r). It has been reported that isolated embryonic day 16.5 oocytes cannot grow beyond 25  $\mu\text{m}$ <sup>27</sup> due to the absence of granulosa cells, which are involved in estrogen biosynthesis. Consistent with isolated embryonic day 16.5 (e16.5) oocytes, the developmental stage of most of the oocyte-like cells could not progress beyond 25  $\mu\text{m}$ , which is probably a consequence of the absence of E2 in the cultures<sup>28</sup>. Collectively, our data suggested that the putative PGCs could initiate sex-specific development and differentiate further into more mature gametes.

Compared to somatic cells, germ cells are distinguished by their ability to switch from mitotic cell division to meiotic division<sup>25</sup>. This change is required for gamete formation. The replication of germ cells to produce oocytes for follicle formation requires the expression of genes involved in the initiation of meiosis<sup>24</sup>. Most oocytes progress to the diplotene stage of prophase I and undergo a protracted arrest<sup>25,28</sup>, known as dictyate/diplonema, in the mammalian ovary, which is traditionally termed a germinal vesicle (GV). To resume and complete the first meiotic division, a primary oocyte has to undergo extensive hormone-dependent growth and maturation<sup>25,28</sup>. A GV-like structure was observed in some oocyte-like cells (Figure 3a, g–j). In order to further examine meiosis relative genes, RT-PCR was performed for the meiotic markers synaptonemal complex component (*SCP1* and *SCP3*). *SCP1* protein elongates transversely along the synapsing homologous chromosomes during the pachytene stage of meiosis, and *SCP1* is downregulated when oocytes arrest in the diplotene stage<sup>29</sup>. During the zygotene stage of meiosis, *SCP3* protein begins to elongate axially along the synapsing sister chromatids, and it exhibits complete axial localization and alignment once it reaches the pachytene stage<sup>30</sup>. The results of the RT-PCR showed that *SCP1* and *SCP3* were present in oocyte-like cells (Figure 3q). Immunocytochemistry staining showed that *SCP3* protein (Figure 3n–p) expressed in a few oocyte-like cells (about 4% of oocyte-like cells) suggested that meiotic protein might be activated in these oocyte-like cells. No oocyte-like cells were observed to extrude a structure resembling a polar body, suggesting that these oocyte-like cells fail to complete meiosis I in this study. This could be attributed to insufficient maturation of the oocyte-like cells.

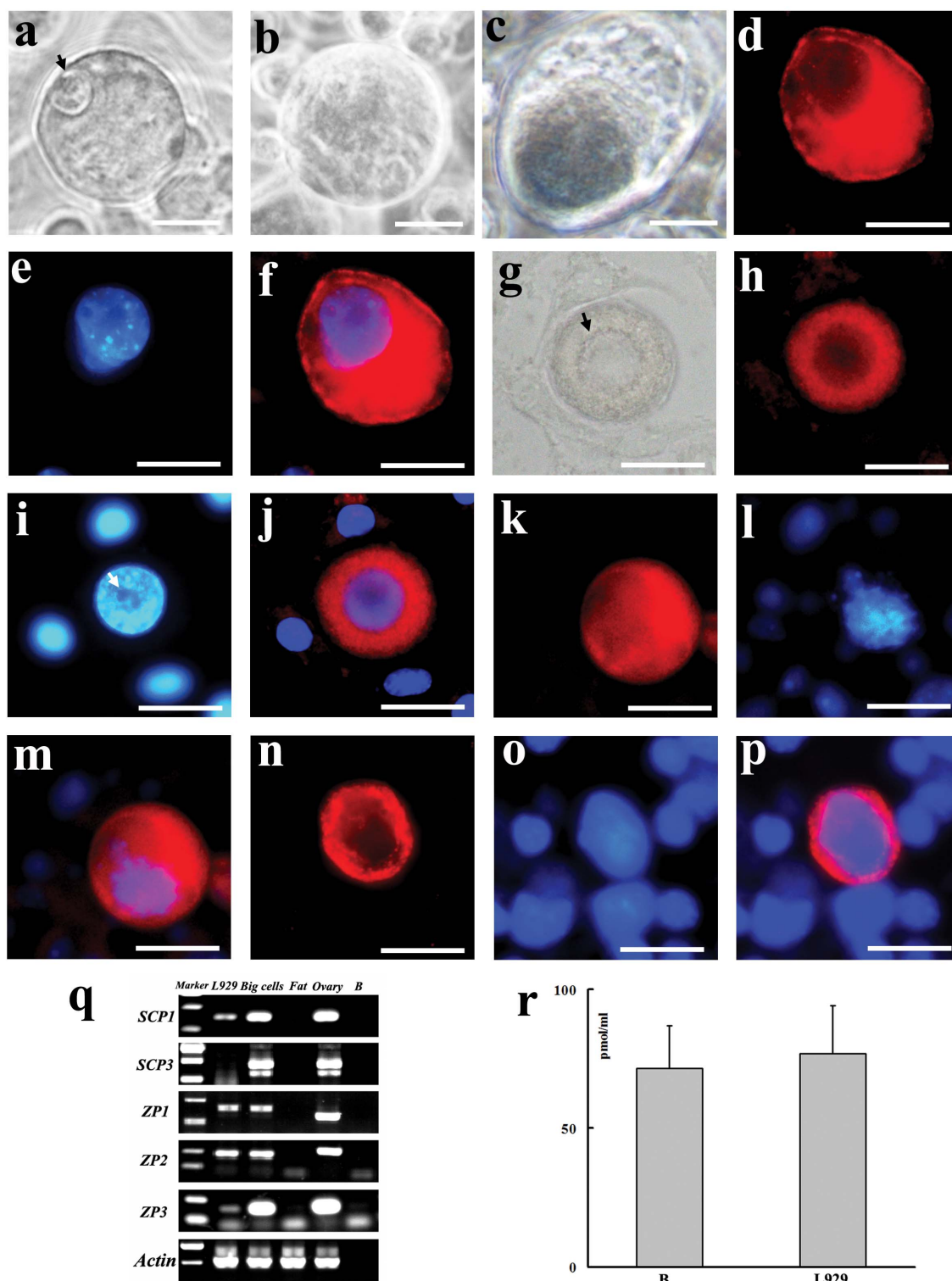
**Formation of embryo-like structures.** Approximately 20% of the oocyte-like cells did not further mature but underwent spontaneous activation to generate parthenogenetic embryo-like structures. Embryo-like structures at different developmental stage from two-cell stage to

blastocyst stage (Figure 4a–g) were observed in cultures. The size of the blastocyst-like structures mostly varied between 20 and 25  $\mu\text{m}$  in diameter (Figure 4a–d), which is consistent with their original oocyte-like cells. A zona pellucida membrane and polar bodies were not detected around the embryo-like structures. Immunocytochemistry staining for Oct4 (Figure 4h–k, Supplemental Fig. S1), Sox2 (Figure 4l–o), and SSEA1 (Figure 4p–s) further indicated that blastocyst-like structures is similar to early natural embryos<sup>13,25,26</sup>. Consistent with mammalian embryo at very early stage, the expression and distribution of Oct4 protein (Figure 4h–k, Supplemental Fig. S1) was detected in cytoplasm and starts to localize in the nucleus<sup>26,39</sup>. Immunocytochemistry showed that *DAZL* were detected in embryo-like structures, which is consistent with the report that *DAZL* protein (Figure 4t–y) is present in mouse preimplantation embryo<sup>31</sup>. Taken together, the results indicated that the oocyte-like cells might be activated spontaneously to develop into preimplantation embryo-like structures in culture.

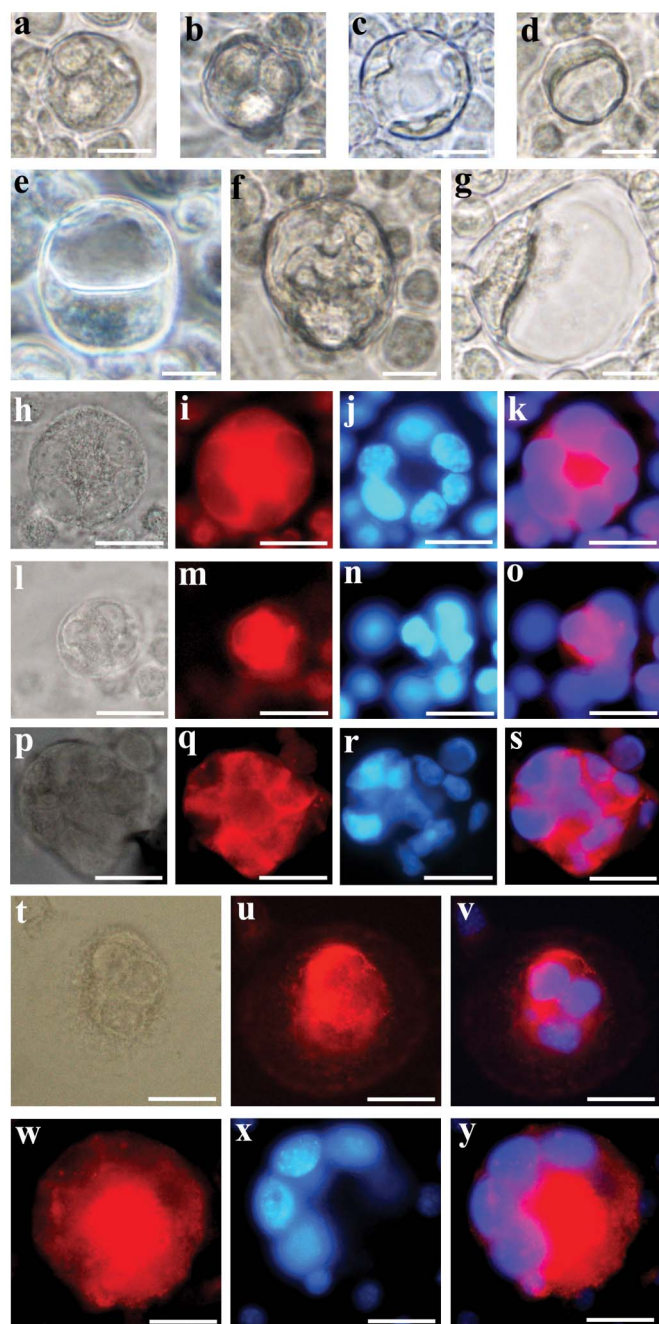
**Formation of teratocarcinomas *in vivo*.** If a mouse male genital ridge at 12.5 dpc is grafted under the kidney capsule, PGCs develop into teratomas containing various differentiated cells as well as undifferentiated stem cells<sup>32</sup>. Parthenogenetic activation of the oocytes can lead to ovarian teratomas<sup>33,34</sup>. To further confirm the formation of germline cells in cultures, we transplanted the L929 cells with germline-like cells subcutaneously into immunodeficient (SCID) mice. Every mouse injected with germline-like cells derived from L929 cells developed a tumor at the injection site in 1 week. The tumors grew rapidly, and the mice were sacrificed after 4 weeks. At this time, the tumors ranged from 1 to 2 cm in diameter. The mice injected with adherent L929 cultures at day 3 after re-plate also did not be observed the formation of tumors until three weeks after injection. The results suggested that the germline-like cells might enhance tumor-initiating potential.

Histological examinations were conducted to analyze the tumor types derived from the germline-like cells *in vivo*. Various differentiated tissues and minimally differentiated tissues from different layers could be observed in the tumors (Figure 5 a–p), including low-differentiation (Figure 5a), sarcoma-like components (Figure 5e, minimally differentiated), epithelial tissues (Figure 5j, minimally differentiated), striated muscles (Figure 5k, mesoderm), bone tissues (Figure 5l, mesoderm), gland-like epithelial tissues (Figure 5m, endoderm), digestive gland-like tissues (Figure 5n, endoderm) and liver-like tissues (Figure 5o, endoderm). However, neural tissues (ectoderm) were not observed in the tumors. The Oct4 (Figure 5d) was detected but *DAZL* (Figure 5c), *Vasa* (Figure 5d), and *SCP3* (not shown) were undetected in the low-differentiation section of the tumors. The Oct4 (Figure 5f), *DAZL* (Figure 5g), and *Vasa* (Figure 5h) were detected while *SCP3* (Figure 5i) were undetected in the sarcoma-like section of the tumors, indicating that germline-like cells might be present in the tumors. Therefore, these results showed that the germline-like cells could give rise to teratocarcinomas *in vivo*, which indicates the germline-like cells similar to natural germ cells in their ability to form tumors.

**Derivation germline-like cells from a single cell.** There are two possible explanations for the spontaneous germline potential of L929 cells. One possibility is that germ cells or ES/EC cells are present among the fibroblast cells. Alternatively, a subpopulation of differentiated cells might reacquire germline potential, due to carcinogenic mutations or epigenetic modifications. Single clone analysis was performed to observe the process of germline-like cell formation from a single differentiated cell. Most of the single cells differentiated and expanded at the beginning (Figure 6a–c). At approximately day 8, round cells appeared in the single-cell cultures (Figure 6d). Upon further culture, the round cells proliferated and formed cell clusters. At day 16, suspending cell aggregates (Figure 6f) and individual germline-like cells (not shown) formed. A subpopulation



**Figure 3 | Formation of oocyte-like cells.** (a) Phase contrast images of an oocyte-like cell with a GV-like structure. (b) A bigger oocyte-like cells. (c) Large oocyte-like cells lacking a zona pellucida membrane. (d-f) Immunocytochemistry showing the expression of DAZL protein in a larger cell. (d) DAZL staining. (e) DAPI staining. (f) A merged image of Dazl protein (red) and DAPI (blue). (g-j) Immunocytochemistry showing the expression of Vasa protein in a bigger cell. (g) Phase contrast images of an oocyte-like cell with a GV-like structure. (h) Vasa staining. (i) DAPI staining (nucleolus, arrow). (j) Vasa/DAPI staining. (k-m) Immunocytochemistry showing the expression of Vasa protein in a larger oocyte-like cell. (k) Phase contrast images of the oocyte-like cell. (l) DAPI staining. (m) Vasa/DAPI staining. (n-p) SCP3 protein was detected in a large cell. (n) SCP3 staining. (o) DAPI staining. (p) SCP3 /DAPI staining. (q) The expression of *ZP2*, *ZP3*, *SCP1*, *SCP3*, and *GDF9* mRNA was detected in these big cells (BC) (>20  $\mu\text{m}$  in diameter). (r) The levels of media estradiol were detectable in culture. Blank (B) medium was used as a control. No statistical difference between the two groups ( $P>0.05$ ). Scale bars = 10  $\mu\text{m}$  in (a-c), 25  $\mu\text{m}$  in (d-p).



**Figure 4 | Formation of structures resembling preimplantation embryos from oocyte-like cells.** Cleavage stage-like embryos and blastocyst-like structures spontaneously generated in cultures (A-G). (a) Two-cell stage embryo-like structure. (b) Three-cell stage embryo-like structure. (c, d) Smaller blastocyst-like structures. (E-G) Bigger blastocyst-like structures. (h-j) Expression of Oct4 protein was detected in a six-cell stage embryo-like structure. (h) Phase contrast images of the embryo-like structure. (i) Oct4 staining. (j) DAPI staining. (k) Oct4/DAPI staining. (l-o) Expression of Sox2 protein was detected in a four-cell stage embryo-like structure. (l) Phase contrast images of the embryo-like cell. (m) Sox2 staining. (n) DAPI staining. (o) Sox2/DAPI staining. (p-s) Expression of SSEA1 protein was detected in an early embryo-like structure. (p) Phase contrast images of the embryo-like structure. (q) SSEA1 staining. (r) DAPI staining. (s) SSEA1/DAPI staining. (t-v) Expression of DAZL protein was detected in embryo-like structure. (t) Phase contrast images of the embryo-like structure. (u) DAZL staining. (v) DAZL/DAPI staining. (w-y) Expression of DAZL protein was detected in a blastocyst-like structure. (w) DAZL staining. (x) DAPI staining. (y) DAZL/DAPI staining. Scale bars = 10  $\mu$ m in (a-g), 25  $\mu$ m in (h-y).

of round cells migrated to another site and formed new clones (Figure 6e), similar to germline migration. Oocyte-like cells (Figure 6g) and embryo-like structures (Figure 6h) could be observed at day 16 in cultures. The results of the RT-PCR showed that Oct4, Sox2, Nanog, Stellar, Nanos3, DAZL, and Vasa were almost undetectable at day7 but were detected at day 16 (Figure 6i) in cultures. The level of Ifitm3 mRNA was low at day7 but higher at day 16 (Figure 6i) in cultures. Immunohistochemistry staining showed that DAZL (Figure 6j, k) and Vasa expressed in germline-like cells, suggesting their similarity to germ cells.

About 92% of single cells (35/38) had the ability to form clones (>16 cells) and all the single clones could generate germline-like cells within two weeks. The results showed that single differentiated cancer cells have the ability to generate germline-like cells, suggesting that germline-like cells could be generated from a single somatic tumor cell.

## Discussion

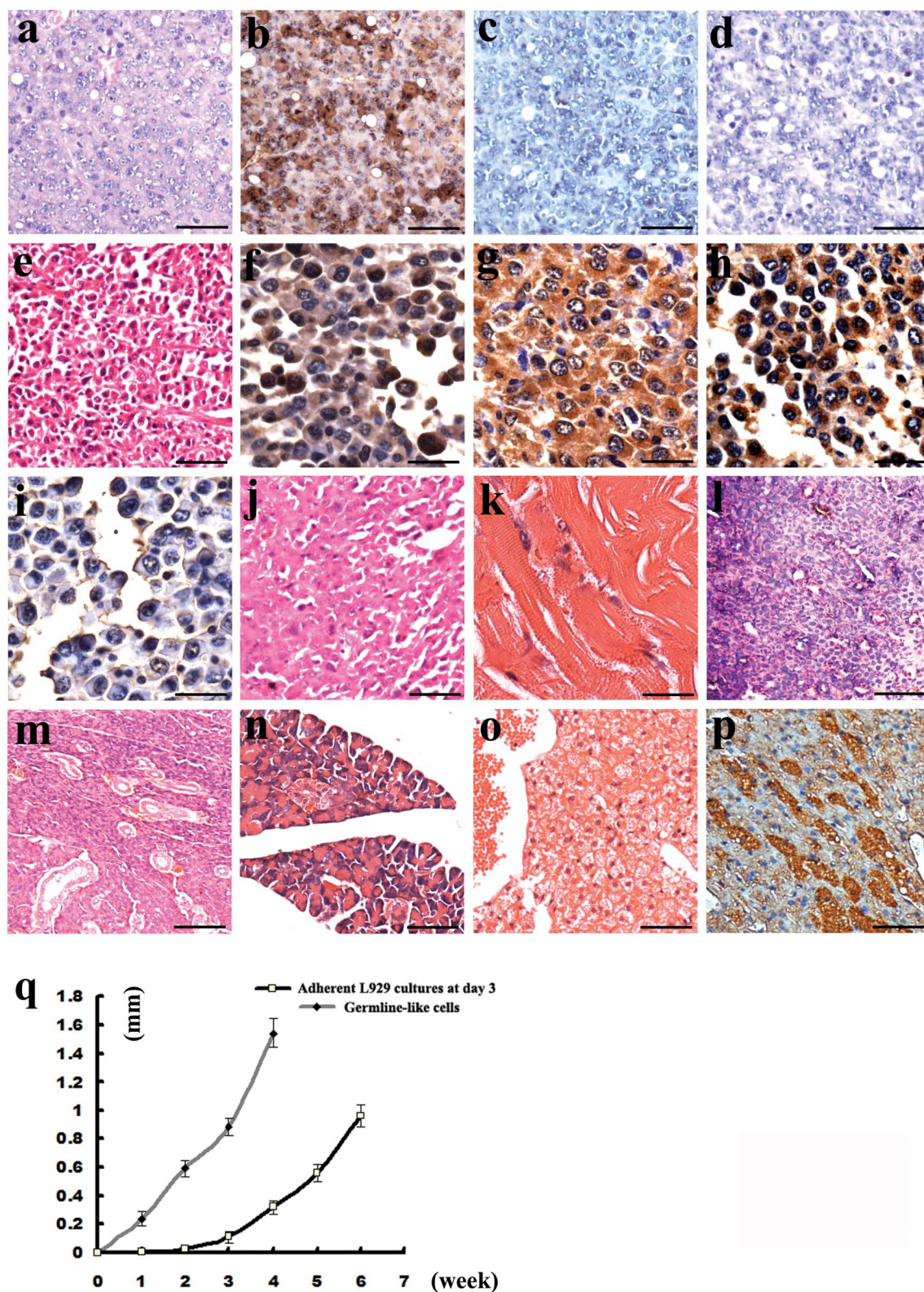
This *in vitro* study showed that the L929 cells could generate early germline-like cells and oocyte-like cells, and the later develop into parthenogenetic embryo-like structures. *In vivo*, the germline-like cells derived from L929 cells could cause teratocarcinomas. These findings indicated that a subpopulation of cells, functioning as germ cells, might be generated from mouse fibrosarcoma L929 cells, even at the single cell level.

The germline potential of L929 cells was similar to that of mouse bone marrow-derived cells<sup>21</sup>, fetal porcine skin stem cells<sup>13,14</sup> adult rat pancreatic stem cells<sup>15</sup> and newborn mouse skin<sup>16</sup> in specific condition. However, we further established that the possible link between the activation of germ cell potential and carcinogenesis. Our previous study<sup>17</sup> showed that germ cell potential could be activated by applying a carcinogen, 3-methylcholanthrene, while the L929 cells were malignantly transformed by long-term culture, thereby suggesting that the activation of germ cell potential is likely to a common event in tumors. Germline-cell formation during carcinogenesis might endow cancer with center malignant features, including immortality, independence, invasiveness, immune evasion, hypomethylation, survival and metastatic capacity<sup>1,10,11</sup>. The present study suggested that the germline-like cells might favor the initiation of tumors, however, further study will be required to further address whether germline-cell formation play important roles in the malignant behaviors. Therefore, it is possible that the germline cells derived from cancer cells might provide potential targets for tumor biology, diagnosis and therapy.

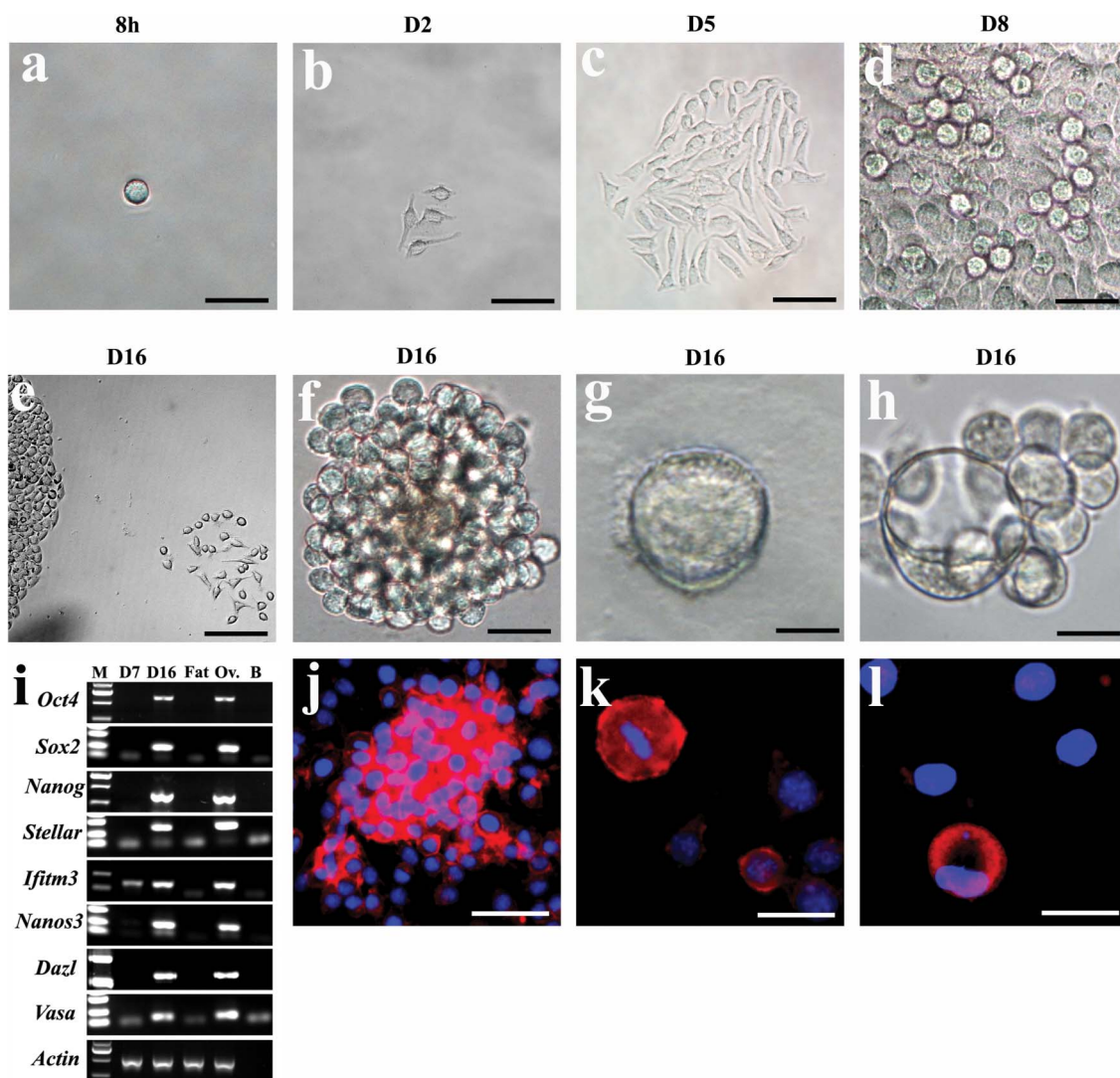
The formation of germline-like cell and embryo-like structures from L929 mimics the process of gametogenesis and fertilization. Therefore, our findings might provide experimental evidences for the idea that there are specific gametogenic or fertilization-related phenomena involved in tumor formation<sup>1,3,34</sup>, and the results possibly shed new light on the century-old question of why there are striking similarities between tumors and germ cells (and their embryonic derivatives)<sup>1,3,35</sup>.

In this study, teratocarcinomas could be generated from L929 cells, indicating that teratocarcinomas might originate also cells derived from adult subcutaneous areolar and adipose tissue, which seemed to extend and provide further supports for our previous reports that teratomas/teratocarcinomas might originate from adult somatic-derived tissue<sup>17,36</sup>.

It is thought that teratomas/teratocarcinomas/germline tumors originate from germline cells or resident ES cells in adult tissues<sup>4,37</sup>. However, the germline potential of a single somatic cell suggested that soma-to-germline transformation might be activated in tumor cells. A similar soma-to-germline transformation was observed in *C. elegans*<sup>38</sup>, which has led some researchers to propose that the acquisition of germline characteristics by somatic cells might contribute to increased fitness and survival<sup>10,38</sup>. However, extensive strong



**Figure 5** | Pathological features and marker expression of tumors derived from L929 cells with germline-like cells. (a-d) Pathological features of the poor-differentiated section and germline relative markers. (a) Pathological features. (b) Oct4. (c) DAZL. (d) VASA. (e-i) Pathological features of sarcoma-like section and expression of germline relative markers. (e) Pathological features. (f) Oct4. (g) DAZL. (h) VASA. (i) SCP3. (j) showing epithelium-like and sarcoma-like pathological features. (k-o) showing the well-differentiated phenotypes in the tumors. (k) Striated muscles. (l) Bone-like tissues. (m) Gland-like tissues. (n) Digestive gland-like tissues. (o) Liver-like tissues. (p) AFP expression in (o). (q) Tumor growth curve from germline-like cells and adherent L929 cultures at day 3. Scale bars = 50  $\mu$ m in (a-e, l, m), 25  $\mu$ m in (f-k, n-p).



**Figure 6 | Generation of germline-like cells from a single cell.** (a) Morphology of a single L929 cell in culture. (b) The isolated L929 cells showed fibroblast-shaped cells at about day 2. (c) Morphology of the single clone at day 5. (d) Round cells appeared above fibroblast-shaped cells in single-cell culture at day 8. (e-h) The cultures were observed at day 16. (e) Round-shaped cells migrated to other site and formed new cell clone. (f) Suspending cell aggregates. (g) An oocyte-like cell. (h) A blastocyst-like structure attaching to a cell aggregate. (i) PCR analysis showed that the expression of *Oct4*, *Sox2*, *Nanog*, *Stellar*, *Ifitm3*, *Nanos3*, *Dazl*, and *Vasa* in cultures at day 7 and day 16 after single-cell culture. Immunocytochemistry showed that *Dazl* positive cells (j, k) and *Vasa* positive cells (l) appeared in cultures at day 16. Scale bars = 10  $\mu$ m in (d, f-h), 20  $\mu$ m in (a-c), 25  $\mu$ m in (j-l), 40  $\mu$ m in (e).

evidences are still needed to further confirm that the germline potential and soma-to-germline transformation are activated during carcinogenesis in mammals.

## Methods

All animal experiments were conducted in strict accordance with the National Institutes of Health Guide for the Care. The protocol was approved by the Committee on the Ethics of Animal Experiments of Huashan Hospital, Fudan University, under permit number SYXK (HU) 2007-0006.

**Cell line and culture.** The L929 cell line (ATCC Number CCL-1, purchased from Shanghai Cell Biology Institute, Chinese Academy of Sciences) were used in this study. Cell line was underwent authentication tests by ATCC. The parent of fibrosarcoma cell line L929 is derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C3H/An mouse. After re-plate, the cultures were cultured continuously for 2–4 weeks without subculture. L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with low glucose (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (PAA) in a 5% CO<sub>2</sub> atmosphere at 37°C. The media were changed twice every week.

**Germline-like cell formation and differentiation.** To observe the formation of germline-like cells in culture, the cells were continuously cultured for 2–4 weeks

without subculture to attain full confluence and high density. Germline-like cells formed spontaneously and underwent further differentiation into larger germline-like cells. Later, these cells generated embryo-like structures. Cultured cells were observed using a microscope.

**RNA Isolation and RT-PCR.** Cultures were collected at different times to study the gene expression. From the oocyte-like cells, approximately 2000 suspended large cells (>20  $\mu$ m in diameter) were collected for RNA isolation and RT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen) and was used as a template for cDNA synthesis using a reverse transcription kit (Invitrogen) according to the manufacturer's instructions. PCR was performed for the amplification of genes using cDNA as a template. All genes, primer sequences, annealing temperatures, and product sizes will be provided when they are needed. Normal subcutaneous areolar and adipose tissues obtained from a three-month-old female mouse were used as negative controls for the detection of mRNA. Ovaries from a new-born female mouse (early marker) and a three-month-old female mouse (later marker) were used as a positive control. The PCR products were resolved using an agarose gel containing EB Stain. Genes and primer sequences were provided in Supplement Table S1.

**Alkaline phosphatase staining.** Cultures with germline-like cells were washed twice with Tris-HCl (pH = 8.2) buffer solution and incubated with alkaline phosphatase (AP) staining solution (Vector Laboratories) in the dark for 2 hours at room temperature. Stained cells were observed by microscopy.





**Immunocytochemistry.** Cultures were disaggregated with trypsin, plated on coverslips placed at the bottom of 6-well plates (at  $5 \times 10^3$  cells per well), and grown for 7 to 20 days. Cells were subsequently fixed with 4% paraformaldehyde for 20 minutes. Cells were incubated for 1 hour in a blocking solution containing  $1 \times$  PBS, 5% BSA, and 0.05% Triton-X-100 (PBS-B) and were incubated overnight at  $4^\circ\text{C}$  with one of several primary antibodies: anti-vimentin (Vim, 7 mg/ml, Chemicon, MAB3400), anti-fibronectin (Fib, 1:200, Chemicon, ab23750), anti-octamer-binding transcription factor 4 (Oct4, 1:200, AbCam, ab19857), anti-stage-specific embryonic antigen-1 (SSEA1, 1:200, Chemicon, MAB4301), anti-Sox2 (1:300, AbCam); anti-Vasa (1:200, AbCam, ab13840), anti-Dazl (1: 150, AbCam, ab34139), or anti-synaptonemal complex component (SCP3, 1:300, AbCam, ab15093). Secondary goat anti-rabbit or mouse antibody conjugated to Cy3 (Jackson) was used for fluorescent detection. Cell nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Invitrogen).

**Flow cytometry.** Cells were dissociated with 0.25% trypsin/EDTA, neutralized with DMEM with 10% FBS, washed twice with PBS and resuspended in PBS. Aliquots of  $1 \times 10^6$  washed cells were stained with either PE anti-SSEA-1 (20  $\mu\text{l}$ , BD, 560142) or PE mouse IgM isotype control (20  $\mu\text{l}$ , BD, 555584), incubated in the dark for 20 min on ice, washed twice with  $1 \times$  PBS and analyzed by flow cytometry. For intracellular staining of Vasa protein, cells were permeabilized in 250  $\mu\text{l}$  fixation/permeabilization solution (Invitrogen) on ice for 20 minutes and were stained with rabbit anti-Vasa antibody (Abcam, ab13840) for 1 hour on ice. FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratory) was used as a secondary. Rabbit IgM isotype protein (Abcam) was used as a control. Three different samples were assayed for every antibody. The mean of the triplicates was used in all subsequent analyses.

**Hormone measurements.** The culture medium of the L929 cells was collected when the medium was changed after 3 days. Blank medium (DMEM with 10% FBS) was used as a control. The concentrations of estradiol (E2) and chorionic gonadotropin (CG) in the medium were measured quantitatively using an electrochemiluminescence immunoassay (Roche) by the Department of Clinical Laboratory Medicine, Huashan Hospital, China. Ten samples and ten blanks were analyzed.

**Tumor formation and analysis.** The later germline-like cells showed little to no adherence. The suspended and low-adherent cells were collected by washing the cultures with medium and were injected subcutaneously into six 4-week-old immune-deficient mice (about 10000 cells per mouse). The adherent L929 cultures (about 10000 cells per mouse) at day 3 after re-plate were injected subcutaneously into six 4-week-old immune-deficient mice. Two mice were injected with PBS as a control. All mice formed tumors within one week. Mice were sacrificed at 4 (germline-like cells) or 6 (adherent L929 cultures) weeks. The control mice did not form tumors at all within their lifespan. The tumors were removed and fixed in 10% neutral-buffered formalin for 24 hours and were embedded in paraffin wax for analysis. Sections of the tumor tissues were stained using routine hematoxylin and eosin (H&E) staining or antibodies for Oct4 (AbCam), Vasa (AbCam), DAZL (AbCam), SCP3 (AbCam), or  $\alpha$ -fetoprotein (AFP, 1:200, R&D, MAB1368). Immunodetection was performed using the SP kit (Zymed). The counterstain of preference for nuclear details was hematoxylin.

**Single cell cloning.** Single cells were plated in 96-well plates and incubated at  $37^\circ\text{C}$  for 8 hours, and cells were adherent to the plate. The 96-well plates were observed by microscopy, and only one adherent cell in each well was chosen. The single cell proliferated and formed a cell clone. The sister cultures from single cells were collected at day7 and day16 for the analysis of RT-PCR or immunocytochemistry.

**Statistical analyses.** Data were analyzed by t-test to identify statistical differences between groups using SPSS 10 analysis software. The results were considered significant at  $P < 0.05$ .

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## Acknowledgments

We thank Ming Guan, Xiaofei Jiang, Min Li, Ming Xu, and Yong Lin for encouragement and support. This work was supported by the grant from the National Clinical Key Subject of China, the Natural Science Foundation of China (No. 30801321), the Research-Scholar Foundation of Shanghai Medical College, and the Medical Key Discipline of Shanghai.



### Author contributions

Liu C and Lu Y wrote the main manuscript text, Ma Z, Hu Y, Jiang G, Liu R and Hou J prepared figures 1-6. All authors reviewed the manuscript.

### Additional information

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

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

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**How to cite this article:** Ma, Z. *et al.* Spontaneous generation of germline characteristics in mouse fibrosarcoma cells. *Sci. Rep.* 2, 743; DOI:10.1038/srep00743 (2012).