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Germline traits of human hepatoblastoma cells associated with growth and metastasis

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ABSTRACT

Genes that are specific to germline and embryonic development can be activated in many tumors. Here, we show that germline traits that are present in human hepatoblastoma cells might be associated with the malignant behaviors of these tumor cells. In culture, single human hepatoblastoma cells differentiated into germ cell-like cells, which further developed into oocyte-like cells and formed parthenogenetic blastocyst-like structures. The germ cell-like cells and their embryonic derivatives from hepatoblastoma cells may favorably give rise to xenograft tumors with embryonal/germline traits and intrahepatic metastasis. These findings suggest that germline potential can be spontaneously activated in human hepatoblastoma cells and it might be important for tumor formation and metastasis.

1. Introduction

The processes of embryonic/germ cell and tumor development share important similarities, including immortalization (which is involved in transformation), invasion, induction of meiosis (which leads to aneuploidy), migration (which contributes to metastasis), demethylation, induction of angiogenesis, down-regulation of the major histocompatibility complex (which contributes to immune evasion) and expression of chorionic gonadotropin [1,2]. The germline-related genes, or the so-called cancer testis (C/T) antigens (~40 identified so far), can be activated and frequently co-expressed in many tumor types [1,2]. Based on the similarities between embryonic/germ cell and tumor development, early and contemporary pathologists have proposed that a specific gametogenic or fertilization-related phenomenon occurs during tumor formation and that it might contribute to the tumor's central malignant characteristics [1,2]. Lloyd J. Old further proposed that activation of germline genes in tumors might reflect activation of the silenced gametogenic program in somatic cells, which may be one of the driving forces of tumorigenesis [1,2].

Recently, Janic et al. revealed that germline traits are necessary for tumor growth [3], suggesting that these traits are indeed associated with some of the malignant characteristics of tumors [3,4]. The study led to an interesting question of which type of cells inside a tumor contributes to the germline traits. To answer this question, the hypotheses of Lloyd J. Old need to be revisited [1,2]. Our previous study showed that mouse bone marrow-derived cells could form teratomas and develop into oocyte-like cells in vitro after induction with a carcinogen [5,6], indicating the possibility of germ cell formation in a tumor cell population. Subsequently, we showed that germ cell-like cells can be derived from cell lines that undergo malignant transformation in long-term cultures [7,8]. Moreover, a single L929 cell (mouse fibrosarcoma cell line) has the ability to transform into germ cell-like cells [7]. Therefore, we sought to further investigate whether gametogenic or embryonic-related differentiation occurs in human tumors and the significance of this process in the malignant behaviors of tumor cells.

Hepatoblastomas represent one of the most common malignant pediatric liver neoplasms. Hepatoblastomas contain various admixed teratomatous elements and express extensive embryonic antigens [9]. In this study, the hepatoblastoma cell line Huh6 [10] was used to investigate the occurrence of germ cell-like cells in tumor cell populations. The results indicated that gametogenic or embryonic-related differentiation was activated in Huh6 cells and that this differentiation might play important roles in the growth and metastasis of xenograft tumors that are derived from Huh6 cells.

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2. Materials and methods

All animal experiments were conducted in strict accordance with the National Institutes of Health Guide for the Care.

2.1. Cell line and culture

The Huh6 cell line (purchased from Shanghai Cell Biology Institute, Chinese Academy of Sciences) was isolated from a human infant patient with liver tumor-hepatoblastoma [10] and maintained in Dulbecco's modified Eagle's medium (DMEM) containing low glucose (Invitrogen, Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS, PAA Laboratory) in a 5% CO2 atmosphere at 37 °C. In this study, a subpopulation of Huh6 cells was continuously cultured for 2–6 weeks without subculture to investigate their germline potential.

2.2. Single cell cloning

The Huh6 cells were plated at a density of 0.5 cells/well in 96-well plates and incubated at 37 °C for 8 h to allow cell attachment. Every well was observed by microscopy, and the wells that contained a single cell were selected. Upon further culture, the single cells proliferated and generated single-cell clones.

2.3. Alkaline phosphatase staining

Huh6 cultures were washed twice with a Tris–HCl (pH = 8.2) buffer solution and incubated with alkaline phosphatase (AP) staining solution (Vector Laboratories) in the dark for 45 min at room temperature. The stained cells were observed by microscopy.

2.4. RNA isolation and RT-PCR

The cells were collected at different time points during culture. Total RNA was extracted using Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Invitrogen) to remove residual genomic DNA. The RNA from each sample was used as a template for cDNA synthesis using the reverse transcription kit (Invitrogen), according to the manufacturer's instructions. Normal hepatic tissue surrounding the liver tumor and ovary tissue surrounding the ovary tumor were used as a negative and positive control, respectively. Embryonic stem (ES) cells were used as a positive control for the early germ cells. The genes were amplified by PCR using cDNA as a template. All genes, primer sequences and product sizes are listed in Table S1.

2.5. Immunochemistry

Cultures were disaggregated with trypsin, plated at 5×10^3 - cells/well and grown on coverslips placed at the bottom of each well for 5–25 days. The cells were subsequently fixed with 4% paraformaldehyde for 20 min. After blocking for 40 min in 1× PBS containing 5% BSA and 0.05% Triton-X-100, the cells were incubated overnight at 4 °C with various primary antibodies, including anti-octamer-binding transcription factor 4 (Oct4; 1:200; rabbit; AbCam; Ab19857), anti-Sox2 (1:200; rabbit; AbCam), anti-c-Kit (1:80; rabbit; AbCam), anti-Vasa (1:200; rabbit; AbCam), anti-deleted in azoospermia-like (DAZL; 1:150; mouse; AbCam), anti-Nanos3 (1:200; rabbit; AbCam), anti- *growth differentiation factor* (GDF9; 1:200; rabbit; AbCam), anti-stage-specific embryonic antigen-3 (SSEA3; 1:200; rat; AbCam) or synaptonemal complex proteins (SCP3; 1:400; rabbit; AbCam). Secondary antibodies that are conjugated to the Cy3 (Jackson) fluorescent dye were used for detection. The cell nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Invitrogen).

2.6. Hormone measurements

Three days after the media was changed, the supernatant was collected and assayed by the Department of Clinical Laboratory Medicine, Huashan Hospital, China for the level of estradiol (E2) using an electrochemiluminescence immunoassay (Roche). Blank medium (DMEM with 10% FBS) was used as a control. Each sample was detected in triplicate, and the average was used in all subsequent analyses.

2.7. Xenograft tumor formation and analysis

The suspended cultures (containing germline-like cells and embryonic-like structures, approximately 1000 cells/mouse) and differentiated Huh6 cells (mainly Huh6 cells, approximately 1×10^6 cells/mouse) from 7 days of subculture were collected and injected subcutaneously into five 4-week-old immune-deficient mice (female BALB/c-nude mice). PBS buffer was injected into two mice as a control. The mice that were injected with the suspended germ cell-like cells were sacrificed after nine weeks, and the mice that were injected with the Huh6 cells were sacrificed after 16 weeks. Tumors were collected at the times of sacrifice. No tumors were observed in the control mice. The xenograft tumor sections were stained with hematoxylin-eosin (H&E) and analyzed for the expression of Oct4, c-Kit, Vasa, Dazl, Hepatocyte (Hep; 1:200; mouse; DAKO) and AFP (1:100; mouse; AbCam) proteins.

2.8. Metastatic tumor formation and analysis

Huh6 cells at day 20 of subculture (containing Huh6 cells and germ cell-like cells, approximately 1×10^6 cells/mouse) were collected and injected subcutaneously into fifteen 4-week-old female BALB/c-nude mice. PBS was injected into five mice as a control. The mice that were injected with the Huh6 cultures were sacrificed after one, two, and six weeks. The mice that were injected with PBS were sacrificed after ten months. Livers were collected from the mice at the times of sacrifice. The xenograft tumor sections were stained with H&E and analyzed for the expression of Oct4, c-Kit, Vasa, Dazl, Nanos3, Sox2, Hep and AFP proteins.

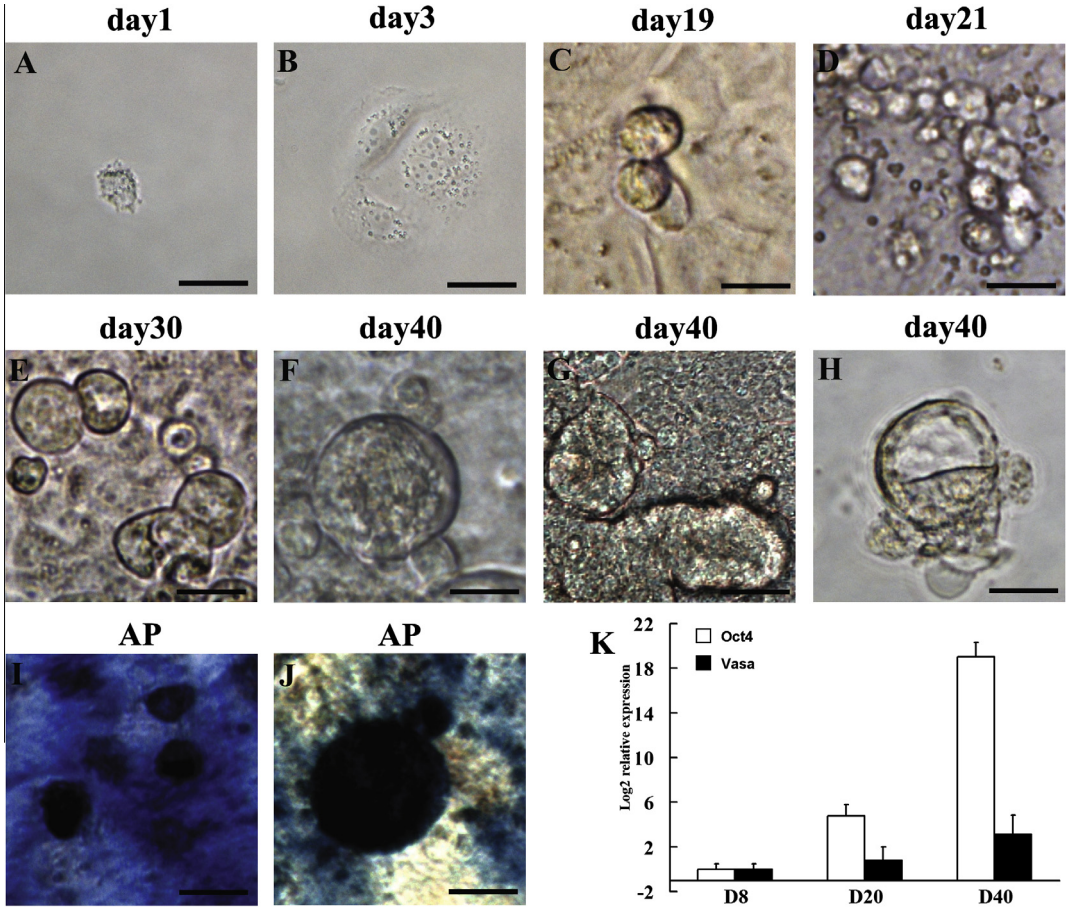
2.9. Statistical analyses

Using SPSS 10 software, the data were tested with the t-test to determine whether significant differences existed between the groups. The differences were considered significant if $P < 0.05$.

3. Results

3.1. Generation of germ cell-like cells from a single Huh6 cell

The Huh6 cell line was derived from a human infant hepatoblastoma [10] and it expresses the hepatic tumor-associated genes CK8, CK18, alpha-fetoprotein (AFP) and E-cadherin (E-cad) (Supplement Fig. S1), which is consistent with the features of hepatoblastoma cells. The cell line was used to investigate the appearance of cells with germline characteristics in hepatoblastoma. Single cell clones were cultured to test their ability to generate germ cell-like cells. These cells showed an epithelial-like shape, and they were able to proliferate and form clones (Fig. 1A and B). After 15–40 days of culture, round cells spontaneously appeared in the single cell colonies (Fig. 1C). Cell aggregates, larger germ cell-like cells, embryo-like structures and EC cell-like clusters were



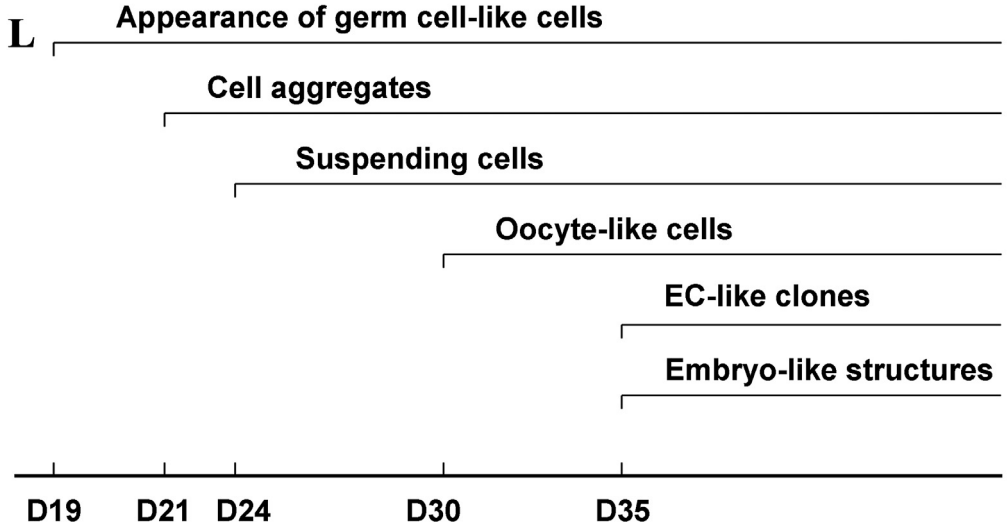


Fig. 1. Derivation of germ cell-like cells from a single cell. (A) The morphology of a single Huh6 cell in a 96-well plate. (B) Proliferation of the cell from (A) on day 3 of culture. Appearance of germ cell-like cells (C), cell aggregates (D), larger germ cell-like cells (E), oocyte-like cells (F), EC-like colonies (G) and embryo-like structures (H). (I and J) The putative germ cells stained positive for AP. (K) Quantitative RT-PCR analysis of *Oct4* (white) and *VASA* (black) on day 8, 20 and 40 of culture. (L) The time course of the generation of germ cell-like cells and their derivatives from a single Huh6 cells. Scale bars = 10 μm.

subsequently observed (Fig. 1D–H). A subpopulation of germ cell-like cells (approximately 10%) detached from the plate and floated in the medium. The germ cell-like cells stained positive for AP (Fig. 1I and J). A single clone analysis showed that approximately 75% of the single differentiated Huh6 cells (15/20) proliferated and formed cell clones. All of the single cell clones (15/15) could generate germ cell-like cells within two months, despite variations in the required length of time between single cells. During the course of single clone expansion, Oct4 and Vasa mRNA levels increased abruptly in the cultures (Fig. 1K). The time course experiment showed that the germ cell-like cells and their derivatives appeared at different time points (Fig. 1L). These results suggest that the germ cell-like cells were generated from a single, epithelial-like cell.

3.2. Characteristics of germ cell-like cells

We further identified the germ cell-like cells according to the characteristics of natural germ cells. The early germ cell-like cells ranged from 5 to 10 μm in size, had a high nucleus-to-cytoplasm ratio (Fig. 2A) and could form additional cell aggregates (Fig. 1D); these morphological features resemble germ cells [11,12]. In addition, germline-specific markers were used to validate the putative germ cells. Similar to natural germ cells [13], the early germ cell-like cells were positive for alkaline phosphatase (AP) activity (Fig. 2B and C) and Oct4 (Fig. 2D), DAZL (Fig. 2E) or Vasa expression (Fig. 2F). Germ cell specification involves a series of well-orchestrated steps that require the concurrent up- and down-regulation of specific markers [13,14]. As expected, the expression of specific genes that are related to germ cell formation was detected in the Huh6 cell cultures, including *Oct4*, *Stellar*, *Sox2*, *TERT*, *Nanog*, *Fragilis*, *Blimp1*, *Nanos3*, *c-kit*, *DAZL* and *Vasa* (Fig. 2K). These results suggest that the germ cell-like cells were similar to natural germ cells in morphology and gene expression.

Approximately 15% of the germ cell-like cells did not self-renew. Instead, they grew gradually into round or ovoid cells that morphologically resembled primary oocytes. Half of the oocyte-like cells floated in the medium. Approximately 95% of the oocyte-like cells ranged from 20 to 40 μm in diameter, while a few of them grew larger than 40 μm. Approximately 5% of the large cells were enclosed in a membrane that resembled zona pellucida (ZP) (Fig. 2G). These large cells also expressed Vasa (Fig. 2H) and DAZL (Fig. 2I), further demonstrating their similarity to oocytes. The synaptonemal complex proteins SCP1 and SCP3 play important roles in meiosis [13]. The detection of SCP3 by immunocytochemistry (Fig. 2J) indicated that meiosis-related genes could be activated in some germ cell-like cells. Organized structures similar in morphology to ovarian follicles were not detected. However, a low level of estradiol could be detected in the medium (Fig. 2L), which supported further growth of the oocyte-like cells above 25 μm [13]. The results of the RT-PCR revealed the expression of oocyte-specific genes in the cultures (Fig. 2M), including *zona pellucida* (*ZP1*, *ZP3*), *GDF9*, the meiotic marker genes (*SCP1* and *SCP3*) and the key genes that are involved in estrogen biosynthesis (*ste-*

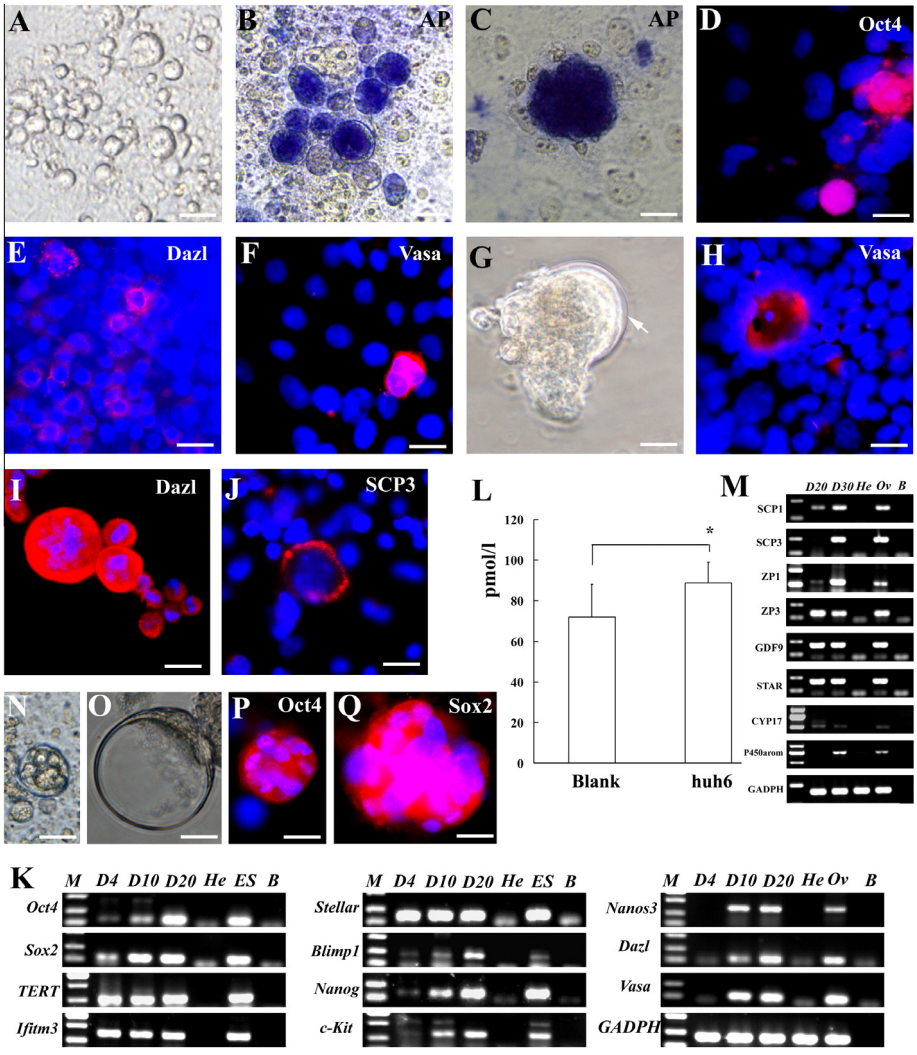


Fig. 2. Characteristics of germ cell-like cells. (A) Small round cells appeared above the Huh6 cells. (B) Germ cell-like cells were positive for AP. (C) Germ cell-like aggregates were positive for AP. (D–F) Small germ cell-like cells were positive for germline markers. (G) An oocyte-like cell with a visible zona pellucida-like membrane (arrow). (H–J) Oocyte-like cells were positive for germline markers. (K) RT-PCR analysis showed that germ cell-related genes were expressed in the cultures. The DNA size markers (M) are indicated in the first lane. Normal hepatic tissue (He), ES cells and ovary tissue (Ov) were used as controls. D4, D10 and D20 correspond to day 4, 10 and 20, respectively. (L) The levels of estradiol in the media were detectable in the cultures. Blank medium was used as a control. A * denotes a significant difference between the two groups ($P < 0.05$). (M) RT-PCR analysis of the cultures on day 20 and 30. (N) An embryo-like structure. (O) Blastocyte-like structure. (P) Expression of Oct4 in an embryo-like structure. (Q) A morula expressing Sox2. Scale bars = 20 μm.

roidogenic acute regulatory protein, StAR; *P450 17-hydroxylase/17,20 lyase*, *CYP17*; and *P450 aromatase*, *P450arom*). Taken together, these results suggest that the germ cell-like cells from Huh6 cells can commit to female germ cell development in culture.

3.3. Characteristics of embryo-like structures

Most of the putative germ cells did not progress through germline maturity but underwent parthenogenetic activation to generate embryo-like structures. Various stages of these embryo-like structures were observed in the cultures (Fig. 2N and O; Supplemental Fig. 2A–K). The size of these structures varied from approximately 12–80 μm in size, and most of them ranged from 12 to 40 μm (Supplemental Fig. 2A–H), suggesting that their progenitors might have been at different stages of development. The smallest structure was approximately 12 μm in size (Supplemental Fig. 2A), suggesting that it was likely derived from the germ cell-like cells at the stage preceding sex commitment. Zona pellucida-like structures were not observed around the embryo-like structures. Although putative inner cell masses were observed in all of the early blastocyst-like structures, trophectoderm layers were not. Consistent with natural embryos, the early embryo-like structures had an embryonic cytoplasmic-to-nuclear ratio and a compact morphology (Supplemental Fig. 2A, B, D and E), and they expressed AP (Supplemental Fig. 2L and M), Oct4 (Fig. 2P; Supplemental Fig. 2N–Q) and Sox2 (Fig. 2Q; Supplemental Fig. 2R–T). Oct4 expression (Supplemental Fig. 2O–Q) was detected in the cytoplasm and gradually localized to the nucleus over time, similar to mammalian embryos at very early stages [11,15]. Collectively, these results indicate that parthenogenetic embryo-like structures can be spontaneously generated from Huh6 cells in vitro.

3.4. Formation and metastasis of xenograft tumors

Germ cells and their embryonic derivatives can give rise to tumors with embryonal components in vivo [16–19]. Subsequently, we addressed whether the germ cell-like cells show the ability of tumor formation and involve in malignant behaviors of tumor cells in vivo. The germ cell-like cells that were grown in suspension and their derivatives (approximately 1000 cells/mouse) gave rise to local subcutaneous xenograft tumors (Fig. 3A) (5/5 mice) within 5 weeks, whereas the differentiated Huh6 cells that were collected

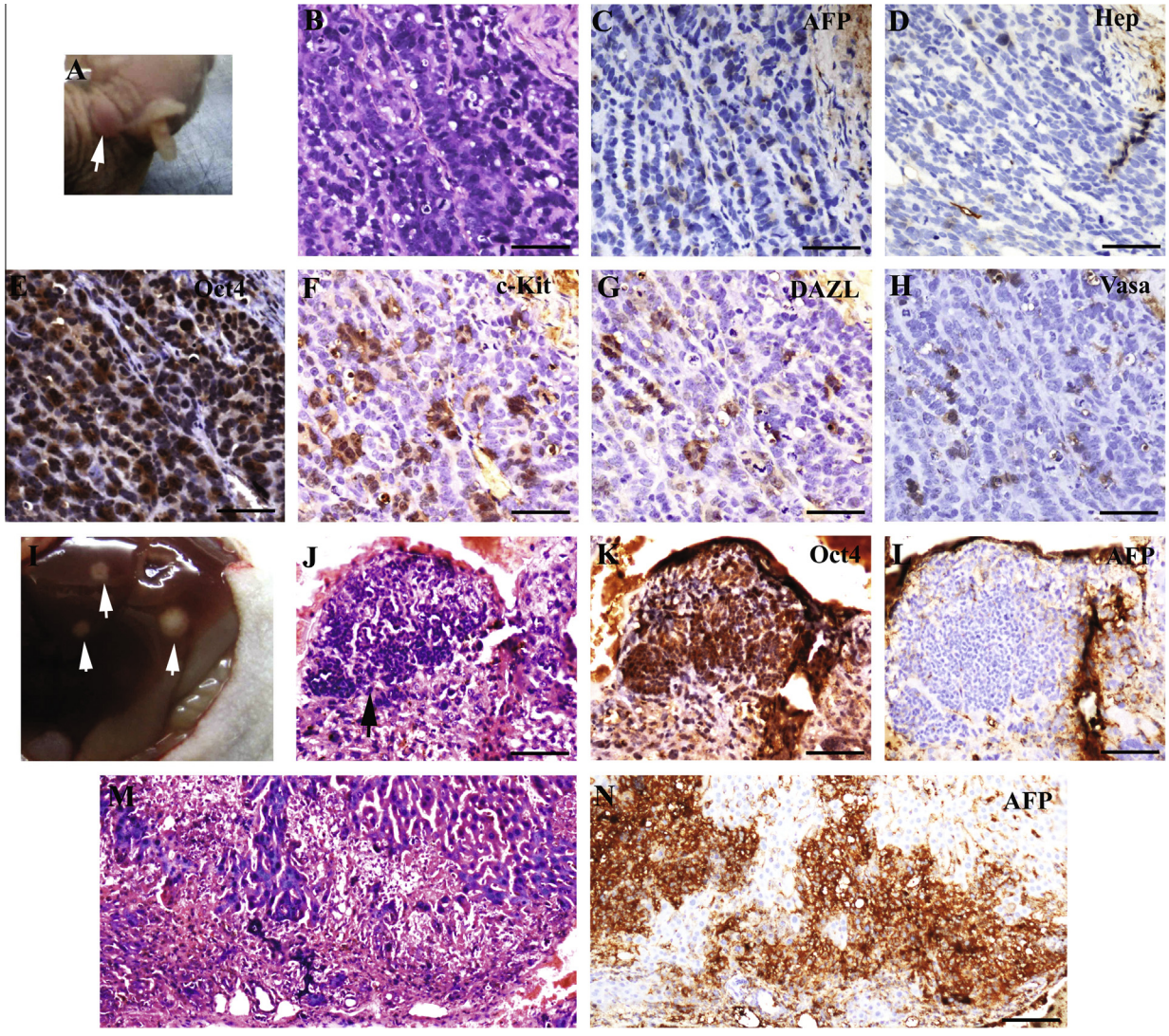


Fig. 3. Characterization of xenograft tumors. (A) Subcutaneous xenograft tumors. (B) Pathological image of the subcutaneous xenograft tumors. (C–H) Immunohistochemical analysis for several markers used to identify the subcutaneous xenograft tumors. (C) AFP; (D) Hep; (E) Oct4; (F) c-Kit; (G) DAZL; (H) Vasa. (I) Intrahepatic metastatic tumors. (J and M) Pathological image of the intrahepatic metastatic tumors. Undifferentiated section (Arrow). (K, L and N) Immunohistochemical analysis for several markers used to identify the intrahepatic metastatic tumors. (K) Oct4; (L and N) AFP. Scale bars = 50 μm.

at day 7 after re-plating (mainly Huh6 cells, approximately 1×10^6 cells/mouse) did not form visible tumors (0/5 mice) even after 16 weeks. Intrahepatic metastases (Fig. 3I) were observed in the mice (4/5) injected with the suspended germ cell-like cells at 9 weeks and in a few of mice (1/5) injected with Huh6 cells at 16 weeks. Other distant metastases were not found. Histological analysis showed that the subcutaneous xenograft tumors mainly consisted of tumor cells with large nuclei and limited cytoplasm (Fig. 3B), resembling embryonal carcinoma in histopathology and protein marker expression (Fig. 3E–H). A few of the local xenograft tumor cells (approximately 5%) expressed the embryonic hepatocyte marker AFP and the hepatocyte marker Hep (Fig. 3C and D), suggesting that these cells differed from the embryonal hepatoblastomas that were derived from the Huh6 cell line by other researchers [20,21]. However, most of local xenograft tumor cells (approximately 80%) expressed the early embryonic/germline related marker, Oct4 (Fig. 3E). In addition, germ cell lineage-like differentiation was observed in the local xenograft tumors (Fig. 3F–H, Supplement Fig. S3). All the intrahepatic xenograft tumors contained undifferentiated cells and tumor cells with the characteristics of embryonal hepatoblastomas (Fig. 3J–N). No local or hepatic tumors were observed in control mice injected with PBS solution. These data indicated that the germ cell-like cells and their derivatives may favorably give rise to xenograft tumors with embryonal/germline traits and intrahepatic metastasis.

3.5. Roles of germ cell-like cells in metastasis

We further investigated the role of germ cell-like cells in the course of metastasis. The mice injected with Huh6 cultures (containing Huh6 cells and germ cell-like cells) were sacrificed at different time points. The earliest microscopic liver metastasis was detectable one week after subcutaneous injection (Fig. 4A). The early metastatic cells were small and morphologically similar to germ cells. They expressed Oct4 (Fig. 4B) but not the other germline markers (Fig. 4C and D), which suggested that these early metastatic cells resembled early-stage germline cells. Proliferation of these early metastatic cells was observed by microscopy (Fig. 4E–

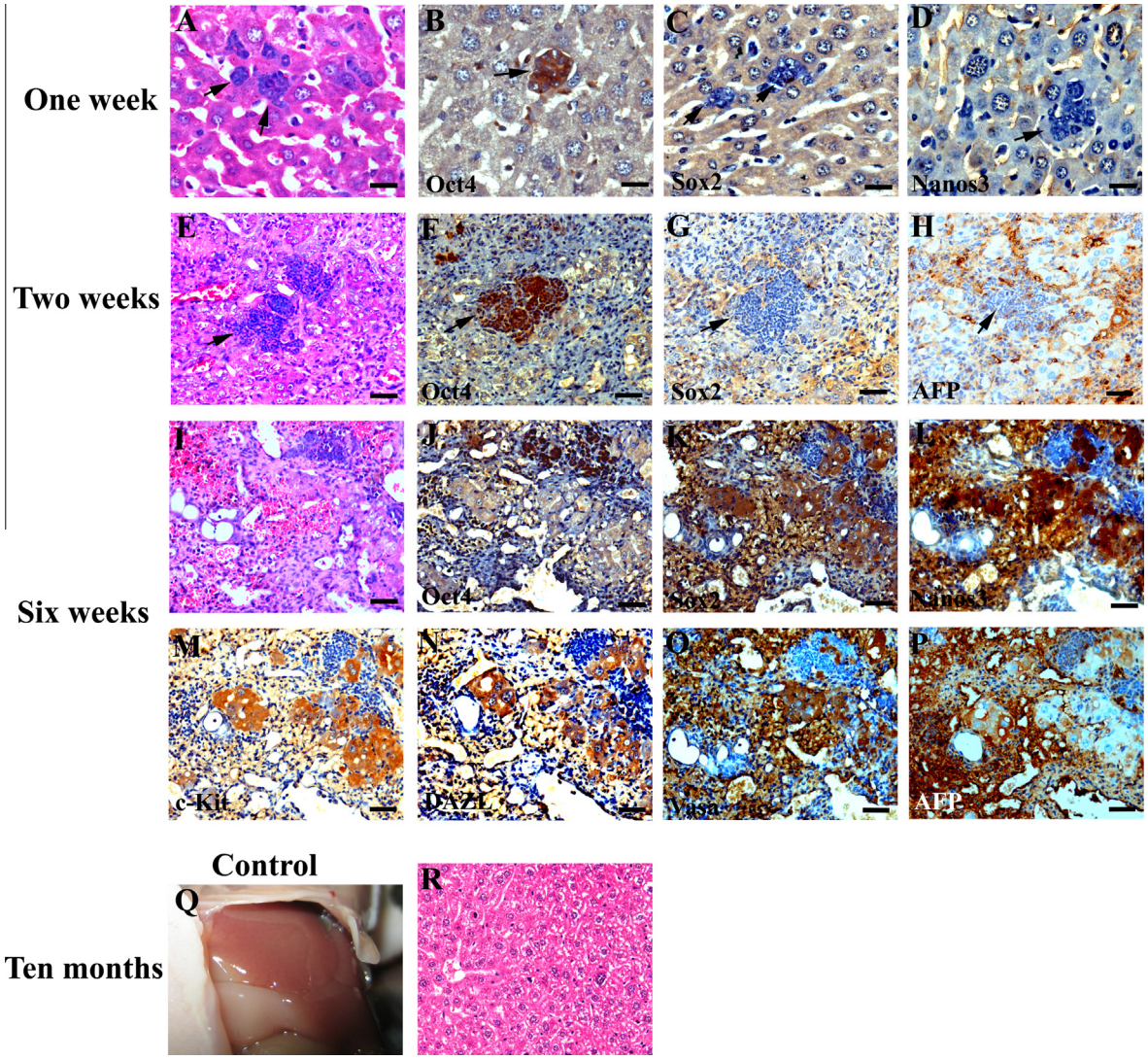


Fig. 4. Intrahepatic metastasis at different time point. (A–D) Mouse hepatic tissue at one week after subcutaneous injection of Huh6 cultures. Pathological image of early metastatic cells (Arrow, smaller cells). Immunochemistry analysis at the one-week time point showing that the early metastatic cells expressed Oct4 (B) but not Sox2 (C) or Nanos3 (D). (E-H) Mouse hepatic tissue at two weeks after injection. Pathological image showing the smaller cells proliferation (Arrow). Immunochemistry analysis showing that the smaller cells expressed Oct4 (E) but not Sox2 (G) or AFP (H). (I–P) Appearance of hepatic and germline differentiation. (L) Immunochemistry analysis showing that the metastatic tumors expressed Oct4 (J), Sox2 (K), Naonos3 (L), c-Kit (M), DAZL (N), Vasa (O), or AFP (P). (Q) Hepatic tissue of mouse injected PBS solution. (R) No abnormal cells were observed in control hepatic tissue under microscopy. Scale bars = 80 μm (A–D); 40 μm (E–P).

H) at the two-week timepoint. After six weeks, the hepatic tumors became megascopic. Differentiation of hepatic tumor cells and more mature germ cell-like cells were observed around the early metastatic cells (Fig. 4I–P). In contrast, no tumor cells were observed in the livers of mice injected with PBS solution, (Fig. 4Q–R). These results showed that the Oct4 positive early germ cell-like cells firstly appeared in the metastatic hepatic tumors, and then differentiated into hepatic tumor cells and later germ cell-like cells. Therefore, these Oct4 positive germ cell-like cells might contribute to the metastasis of Huh6 tumor cells.

4. Discussion

This study shows that the human hepatoblastoma cell line Huh6 can generate spontaneously germ cell-like cells and parthenogenetic embryo-like derivatives at the single cell level in vitro and give rise to xenograft tumors with germline/embryonic traits. These findings indicate that hepatoblastoma cells can undergo germ cell/gamete/trophoblast differentiation in vitro, which might explain why hepatoblastomas possess a strong embryonic/germline nature [1,2]. Compared with normal somatic tissue-derived cells [12,14,22,23], the germline potential in the Huh6 cell populations was activated spontaneously without specific factors, similar to ES cells [10,24,25]. It is possible that carcinogenesis might be a factor that drives the activation of germline potential in somatic cells.

There are two explanations for germ cell formation from Huh6 cells. One explanation is that germ cells, embryonal carcinoma (EC) cells or embryonic stem (ES) cells are all present in a population of Huh6 cells [16,19]. Alternatively, a subpopulation of well-differentiated Huh6 cells can be reactivated to generate germ cells during carcinogenesis. The somatic-to-germline cell transformation that was previously observed in *Caenorhabditis elegans* indicates that the acquisition of germline characteristics by somatic cells might contribute to increased cell fitness and survival [26]. The generation of germ cell-like cells from Huh6 cells at the single cell level suggests that somatic-to-germline cell transformation also occurs possibly in Huh6 cells, which would support the hypothesis that specific gametogenic programs are activated in somatic tumors [1,2]. However, further studies are still required to address the mechanism for the somatic-to-germline cell transformation.

It is believed that germ cell formation might confer some of the central malignant characteristics to tumors, including immortality, independence, invasiveness, immune evasion, hypomethylation, survival and metastatic capacity [2–4]. Janic et al. reported that germline traits are necessary for tumor growth [3,4]. Our current findings further suggest that germ cell-like cells in a population of Huh6 cells might play an important role in the growth of local xenograft tumors and the intrahepatic metastasis. Therefore, the Huh6 germ cell-like cells may represent a potential target for hepatoblastoma diagnosis and therapy. However, in-depth studies are still required to further elucidate the significance of germ cell-like cells in malignant tumors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.050.

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