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Article

Somatic-to-primordial Germ Cell-Like Transformation is Critical in Tumour Initiation of Mouse Breast Tumour 4T1 Cells

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Abstract: It has been proposed that tumourigenicity was an intrinsic feature of embryonic/germ cell developmental axis as well as embryonic/germ cell-related genes play a crucial role in tumourigenicity. Our previous studies indicated that primordial germ cell (PGC)-like potential could be reactivated in tumourigenesis. In this study, 4T1, 168FARN and 67NR cells which originated from the same mouse breast cancer were studied and the results indicated that the acquisition of embryonic/germ cell-like state is essential for tumourigenicity. We further demonstrated that somatic to PGC-like transformation (SPLT) was activated in 4T1 cells and that inhibition of PGC-like cell formation by depleting pluripotency and/or PGC specification-related genes markedly repressed SPLT and the tumourigenicity. Collectively, our findings reveal that tumourigenicity is linked to the acquisition of PGC-like state through SPLT in 4T1 cells, provide new insight into deeper understanding the biological nature of tumours and novel therapeutical strategies for cancer targeting.

Keywords: Tumor initiation; Germ cell traits of tumors; Primordial germ cell-like tumor cells; Somatic to Primordial germ cell-like transformation; Embryonic/germ cell hypothesis of tumor; Breast cancer

Introduction

It is still under debate which phenotype is a driving force in tumour malignant behaviors, such as tumourigenicity. Of note is that embryonal/gametogenesis hypothesis of tumours was postulated at the beginning of tumours identified, which stated that tumours arise from germ cells (missing germ cells or reobtaining the embryonic/germ cell fate of somatic cells) based on the striking similarities between carcinogenesis and embryonic/germ cell development [1-4]. Intriguingly, it has been proposed that tumourigenicity is an intrinsic feature of embryonic/germ cell developmental axis, such as blastomeres, embryonic stem (ES) cells, and early primordial germ cells (PGCs) as well as induced embryonic state-like cells, such as parthenogenesis activation of oocytes, embryonic germ cells (EGCs) and induced pluripotent stem cells (iPS cells) [5-10]. This model postulates that if cells acquiring ES cell or early PGC features occurred in somatic tissues through mechanisms yet to be discovered, tumours might be caused.

More recently, it became increasingly clear that tumour development is highly similar to embryonic/germ cell development in terms of marker expression, pathways involved, behaviors, and immune escape [11-23]. Notably, genes related to embryonic/germ cell developmental axis are essential for tumour formation and progression in addition to those classical oncogenes and tumour suppressor genes, such as Ras, c-Myc, Rb, p53, and PI3K, which are also known to serve as master regulators in reprograming

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or embryonic/germ cell development [15, 16, 19, 22, 24-27]. Of not is that our previous studies showed that embryonic/germ cell-like cells appeared in various types of tumour cells, from PGC-like cells, oocyte-like cells to blastomere-like cells which are associated with tumour formation, liver metastasis, drug resistance and somatic embryo-like life cycle [17, 19, 28-31], providing the possibly cellular basis for the embryonal/gametogenesis-related hypothesis of tumours. Moreover, the activation of embryonic/germ cell-like cell formation appeared in somatic tumour cells can be induced by either chemical carcinogen (3-methyl-cholanthrene, 3-MCA) or *p53* deficiency [18, 28]. Therefore, we postulated that somatic to embryonic/germ cell-like state transformation might be a driving force in tumour initiation. In this study, we revealed that somatic to PGC-like transformation (SPLT) is essential for tumourigenicity of mouse breast cancer 4T1 cells.

Correlation between embryonic/germ cell-like cell formation and tumour initiation

The isogenic cell subpopulations from the same mouse mammary tumour 4T1, 168FARN and 67NR cells were firstly used to investigate which phenotypic properties is essential for tumourigenicity. Our in vivo animal experiments revealed that the three tumour cells had a marked difference in tumourigenicity (Fig. 1a). The mice injected with 4T1 cells formed tumours quickly (Fig. 1a) and developed metastasis [19]. Compared to 4T1 cells, 168FARN cells delayed tumour initiation (Fig.1a) and failed to form obvious metastasis [19]. Although 4T1 and 168FARN cells were different in tumour onset, but their tumour growth rate in vivo was similar (Fig.1a). However, the mice injected with 67NR cells failed to give rise to tumour 6 months after subcutaneous injection (Fig.1a).

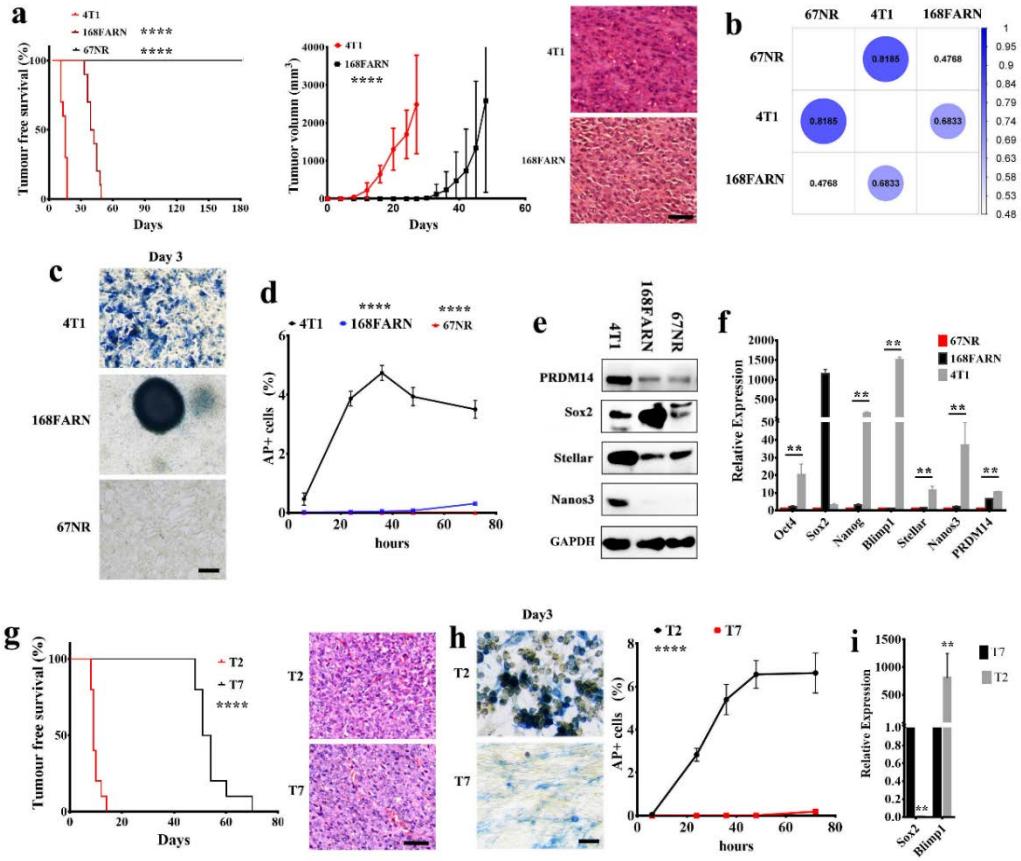

Fig1. Embryonic/germ cell-like cell formation associated with tumour initiation. (a) The tumour-free survival curve showed the difference of 4T1, 168FARN and 67NR cells in tumour initiation potential (left). Tumour initiation and growth curve of 4T1 and 168FARN cells (middle). Bright field image of tumour tissue section with HE staining of 4T1 and 168FARN cells (right). (b) DNA sequencing showed the correlation of genetic changes among the 4T1, 168FARN and 67NR cultures.

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(c) Bright field image showed AP staining in 4T1, 168FARN and 467NR cultures. (d) The plot showed the formation efficiency of AP-positive cells in 4T1, 168FARN and 67NR cultures at different time points. (e) WB results of indicated proteins in 4T1, 168FARN and 67NR cultures. (f) qRT-PCR showed the expression of indicated genes in 4T1, 168FARN and 67NR cultures. (g) The tumour-free survival curve showed the difference of TBMDCs-2 (T2) and TBMDCs-7 (T7) in tumour initiation potential (left). Bright field image of tumour tissue section with HE staining of TBMDCs-2 (T2) and TBMDCs-7 (T7) in mice (right). (h) Bright field image showed AP staining in TBMDCs-2 (T2) and TBMDCs-7 (T7) cultures. The plot showed the formation efficiency of AP-positive cells in TBMDCs-2 (T2) and TBMDCs-7 (T7) cultures at different time points. (i) qRT-PCR showed the expression of indicated genes in T7 and T2 cultures. Scale bar=50 μm (a, c, h). **p<0.01, ****p<0.0001.

The results of DNA and RNA sequencing showed that all three cell lines underwent massive changes in gene sequence and expression (Fig.1b and Fig.S1-5). Considering the fact that the three cell lines also showed a distinct difference in gene expression related to tumours, we postulated that their difference in tumourigenicity possibly attributes to a core phenotypic trait induced by a serial of gene changes. To validate our concept that the embryonic/germ cell state might be the core phenotype, we investigated the difference of the three isogenic cells in embryonic/germ cell traits. Interestingly, the ratio of a subpopulation of cells positive for alkaline phosphatase (AP) staining, a marker of embryonic stem cells and early germ cells [32] was significantly distinct in the three cancer cell cultures (Fig. 1c, d). The AP^+ cells were round in 4T1 cultures (Fig.1c), resembling the PGCs in shape which have the ability to drive tumour and metastasis [19]. In 168FARN cultures, AP^+ cells often formed tightly clusters which were closer to ES cell clones in morphology which could cause tumour but fail to drive metastasis (Fig. 1c). Although there are several gene mutations appeared in the cells (Fig.S1), neither PGC-like nor ES-like state was observed in 67NR cultures (Fig.1c, d), consistent with their low tumourigenicity[33].

The results of qRT-PCR and Western blot (WB) assays further confirmed the difference in embryonic/germ cell-like cell formation among 4T1, 168FARN and 67NR cell cultures. Compared with 168FARN cell cultures, 4T1 cell cultures displayed much higher expression in genes critical for PGC-like state [32] (Fig. 1e, f), such as Stellar, Nanos3 and Blimp1, possibly linking to metastatic ability. However, the Sox2 gene was amplified (Extended-table S3) and highly expressed in 168FARN cells (Fig.1e, f), which might promote the embryonic cell-like state but inhibit PGC-like state [32]. The data of WB showed that some embryonic/germ cell-related genes were also activated in 67NR cells (Fig. 1e), raising the possibility that tumourigenicity is not caused by single ES/PGC-related gene instead is an outcome of ES/PGC-like state re-obtaining through a gene network operated together. The findings indicated that the ability of AP^+ embryonic/germ cell-like cell formation is positively correlated with tumourigenicity among the three cell lines as well as the PGC-like rather than embryonic cell-like properties link to metastatic ability [19].

The similarly positive correlation between the ability of embryonic/germ cell-like cell formation[19] and tumourigenicity was also observed in transformed bone marrow-derived cells (TBMDCs-2 and TBMDCs-7) (Fig. 1g-i). Collectively, one of the most intriguing possibilities is that obtaining the ability of embryonic/germ-cell formation might be a driving force in tumourigenicity.

Activation of somatic cell-to-PGC like transformation (SPLT) in 4T1 cells

The studies of iPS cells provided a direct evidence for the reprogramming in somatic cells and validated that the reobtaining of the ES-like state in somatic cells could confer tumourigenicity. Because numerous studies showed that embryonic state is essential for tumourigenicity, we mainly focused on whether the obtaining of PGC-like properties plays a crucial role in tumourigenicity in a given tumour cell line, 4T1 cells. In essence, the normal PGCs are unipotent and destinated to develop into oocytes/sperms. In order to further confirm the similarities between PGC-like cells and natural PGCs, we analyzed the development of PGC-like cells along with germ cell maturation in morphology and marker expression. Round-shaped cells with varied size and positive for AP staining

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could be observed in 4T1 cells, including PGC-like cells, post-migratory PGC-like cells, and oogonia-like cells, however, the late oocyte-like cells and blastomere-like structures were barely observed in normal culture (Fig. 2a, b), which required certain culture conditions. Of note is that the PGCs can return to EGCs and then give rise to somatic cells under certain conditions (e.g. *PTEN* deletion or *TP53* deletion), thereby providing the basis for spontaneous testis teratomas developed in the animal model with *Pten* or *TP53* deficiency [8, 32]. It is possible that the PGC-like cells returned to somatic state through PGC-EGC-somatic cell-like pathway rather than undergoing further development along with germ cell maturation and parthenogenetic activation in 4T1 cultures.

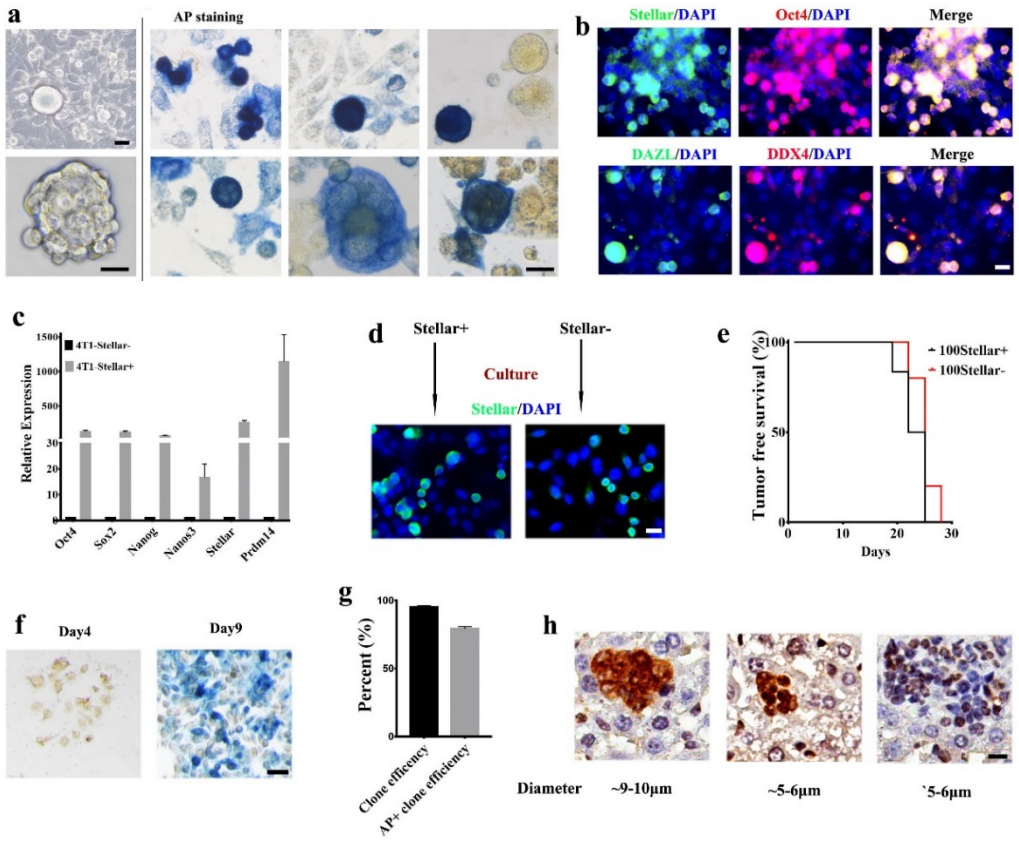

Fig2. A crucial role of PGC-like cell formation in tumour initiation of 4T1 cells. (a) Bright field image of germ cell-like cells and pre-implantation embryo-like structures with or without AP staining. (b) The immunofluorescence showed 4T1culturs stained with indicated antibodies. (c) qRT-PCR showed the expression of indicated genes in 4T1 stellar$^+$ or Stellar$^-$ cells. (d) Both 4T1 stellar$^+$ and Stellar$^-$ cells generated offspring containing stellar$^+$ and Stellar$^-$ cells. (e) The tumour-free survival curve showed the tumour initiation potential in 4T1 stellar$^+$ or Stellar$^-$ cells. (f) Derivation of clone with AP$^+$ cells from AP$^-$ cells at single cell level. (g) The efficiency of clones and clones with AP$^+$ round cells in 4T1 cells at single level within a month. (h) In the hepatic micro-metastasis of 4T1 cells, smaller round cells resembling ES cells which was ~ 5-6 μm in diameter and expressed Oct4 in nucleus could be observed during the derivation of somatic tumour cells from PGC-like cells (~9-10 μm in diameter). Scale bar=10 μm (h), 20 μm (a, b, d), 50 (f). **p<0.01, ****p<0.0001.

We then isolated the Stellar$^+$ (a marker of PGCs, represent PGC-like cells) with FACS analysis in 4T1 cultures to investigate whether PGC-like cells produce somatic tumour cells. Compared to Stellar$^-$ cells, the Stellar$^+$ cells displayed the high expression of the genes related to PGC-like state (Fig. 2c). As expected, the Stellar$^+$ cells switched back to somatic cells after culture instead to the oocyte-like cells (Fig. 2d). Of note, the stellar$^-$ cells (represent somatic tumour-like cells) isolated from 4T1 cultures with FACS could generate PGC-like cells (Fig. 2d). In addition, both 100 Stellar$^+$ cells and 100 Stellar$^-$ cells of 4T1 cultures

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injected to mice displayed similar tumourigenicity potential (Fig. 2e). Consistent with this result, the AP+ round-shaped cells and the AP- somatic-shaped cells also could switch mutually, even at single cell level (not shown). After 12h culture, almost all cells showed somatic morphology and low AP expression in 4T1 single cell culture (not shown). With the long-term culture for a month, AP+ round cells appeared and the ratio of AP- cell to AP+ conversion reached up to 70 percent in 4T1 (Fig. 2f, 2g). In the hepatic micro-metastasis of 4T1 cells, ES-like cells which was about 5-6 μm in diameter and expressed Oct4 in nucleus could be observed during the derivation of somatic tumour cells from PGC-like cells (Fig. 2h), indicating that PGC-like cells generated into somatic tumour cells through the similar way to PGC-EGC-somatic cell conversion in testicular teratomas [8]. Taken together, the findings suggest that somatic-PGC-like mutual transformation appears in 4T1 cells.

A crucial role of SPLT formation in tumourigenicity

To validate the crucial role of PGC-like state in the tumourigenicity of 4T1 cells and the similarities of the PGC-like cells with natural PGCs, we knocked out a serial of genes related with PGC specification in 4T1 cells using CRISPR-Cas9, such as embryonic/germ cell related gene Oct4, Sox2, Nanog or PRDM14 as well as germ cell specific genes DDX4 or DAZL [32, 34]. Upon knockout of any one of these genes, the formation of PGC-like cells was impaired abruptly (Fig. 3a), indicating that the formation of PGC-like cells is likely maintained by a gene network like the nature PGCs [32, 34]. At the single cell level, the efficiency of SPLT was decreased abruptly in 4T1-KO group versus 4T1-control group (Fig. 3b). To further validate the critical role of SPLT in tumourigenicity, we subcutaneously injected these knockout cells into mice. Inhibition of SPLT by knockout of any one of the genes in 4T1 cells abrogated tumour initiating potential (Fig. 3c). Thus, obtaining the SPLT is essential for tumourigenicity of 4T1 cells, and SPLT is governed by a serial of PGC development-related genes. It is possible that the breakdown of SPLT might be a potential strategy for cancer therapy.

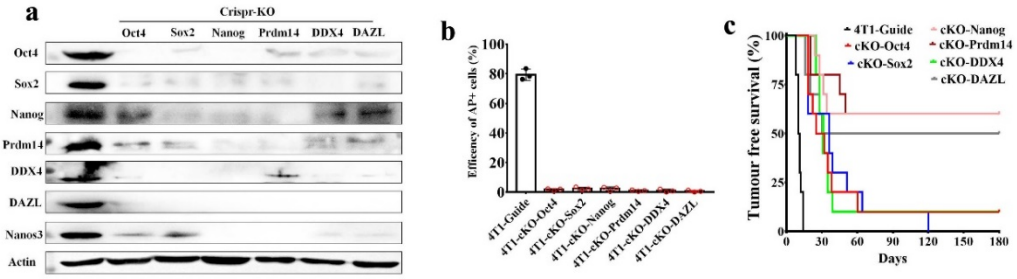

Fig3. A crucial role of PGC-like cell formation in tumour initiation of 4T1 cells. (a) WB results of indicated proteins in 4T1 cultures with different knockout gene. (b) The plot shows the formation efficiency of AP-positive cells in 4T1 cells with different knockout gene versus 4T1 control cells. (c) The tumour-free survival curve showed the difference of 4T1 cells with different knockout gene and 4T1 control cells in tumour initiation potential.

PGC specification pathway orchestrates SPLT

PGC specification is viewed as the first and perhaps most critical event in germ cell development. It was documented that BMP pathway is essential in governing PGC specification [32, 35]. We then investigated whether the pathway of PGC specification regulates the SPLT. We found that DMH2, an inhibitor of activin A receptor type I (Acrv1), which is an essential receptor of BMP pathway in PGC specification, markedly suppressed proliferation and PGC-like cell formation in 4T1 cultures in a dose-dependent manner (Fig. 4a, b). Upon treatment with 8 μM DMH2 for 48h, the AP+ cells were barely observed in 4T1 cultures (Fig. 4b). Consistently, the results of WB showed that DMH2 inhibited the

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Smad1/Smad5 signal pathway [36] (Fig. 4a) which is the key regulator of PGC specification, accompanied by impairing the expression of embryonic/germ cell related genes and germ cell specific genes (Fig. 4a). Of note, the removal of the DMH2 restored the SPLT in 4T1 cells (Fig. 4b). Alteration of gene expression related to PGC specification during the course of DMH2 treatment is correlated with the formation of PGC-like cells (Fig. 4c). After treated with 8 μM DMH2 for 48h, the 4T1 cultures were injected into mice. The animal data showed that the DMH2 treated group versus control group displayed the similar tumourigenicity potential (Fig. 4d), consistent with the fact that SPLT state was restored after DMH2 removal. The findings indicated that BMP pathway, a core pathway of PGC specification [32, 35], might be a master regulator in SPLT.

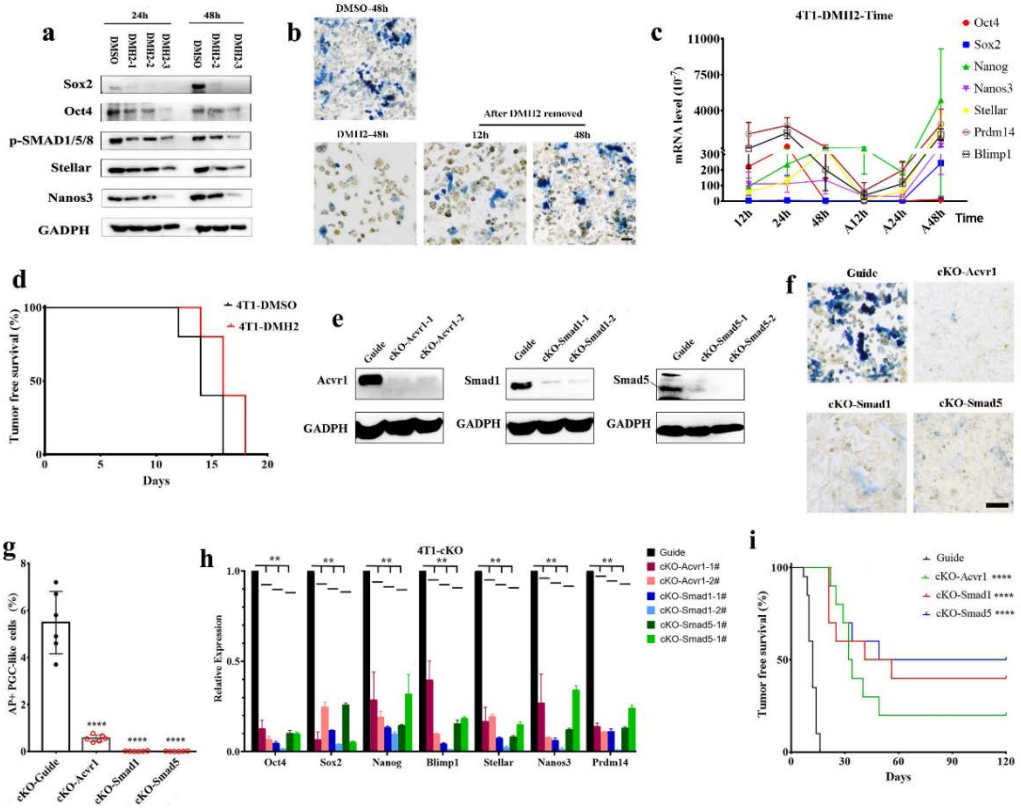

Fig.4 Roles of BMPs pathway in the tumourigenicity of 4T1 cells. (a) WB results of indicated proteins in 4T1 cultures treatment with DMSO (control) or DMH2 at different time points. The concentration of DMH2-1, DMH2-2 and DMH2-3 was 3 μM, 6 μM and 8 μM respectively. (b) Bright field image of AP^+ germ cell-like cell formation in 4T1 cultures treatment with DMSO (control) or DMH2 at different time point. (c) The mRNA level of indicated genes versus GAPDH in 4T1 cell cultures treated with DMH2 (8 μM) at the different time points and after DMH2 removal. (d) The survival curve of tumour-free cells showed tumour initiation potential in 4T1 cultures treated with DMSO (control) or DMH2(8 μM) for 48h. (e) WB results showed that indicated proteins expressed in 4T1 cultures with control or indicated gene knockout. (f) AP staining of 4T1 cultures with control or knockout of indicated genes. (g) The percentage of AP^+ PGC-like cells in 4T1 cells with control or indicated gene knockout. (h) The mRNA level of indicated genes in 4T1 cultures with control or indicated gene knockout. (i) The survival curve of tumour-free cells showed the difference between 4T1-control and 4T1-KO indicated gene in the ability of tumour initiation. Scale bar=50 μm (b, f). **p<0.01, ****p<0.0001.

To further validate the crucial role of BMP pathway, we then investigated the role of the BMP pathway, such as Acrv1 and Smad1/Smad5 in the SPLT of 4T1 cells [32, 35]. After deleting the Acvr1, Smad1 or Smad5 with CRIPR-Cas9 technology, the 4T1 cells displayed the impaired PGC-like cell formation (Fig. 4e-h). Consistently, the data of RT-PCR showed that the genes related to PGC specification decreased robustly after knockout of

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Acvr1, Smad1 or Smad5 (Fig. 4h). The efficiency of SPLC was decreased abruptly in 4T1-KO group versus 4T1-control group (Fig. 4f-h). As a result, the 4T1-KO group showed abrupt decline in tumour initiating potential compared with 4T1-control group (Fig. 4i). These findings further validate that PGC specification pathway [32, 35] governs SPLT, which is essential for tumourigenicity of 4T1 cells.

Roles of Blimp1 in SPLT and tumourigenicity

To further strengthen the concept, we then investigated whether Blimp1, an important downstream target of Smad1/Smad5 serving as one of the core regulators of PGC specification [32, 35], is essential for SPLT. Interestingly, the expression of Blimp1 was higher in 4T1 and T2 than 168FARN and T7 cells (Fig. 1f, 1i), which might contribute to distinguishing the formation of PGC-like state among the cell lines. After deletion of Blimp1 with CRISPR-Cas9, the 4T1 cells showed robust impairment in SPLC and tumourigenicity (Fig. 5a-d). Together, the findings further indicated that PGC specification governs SPLT and tumour initiation in 4T1 cultures.

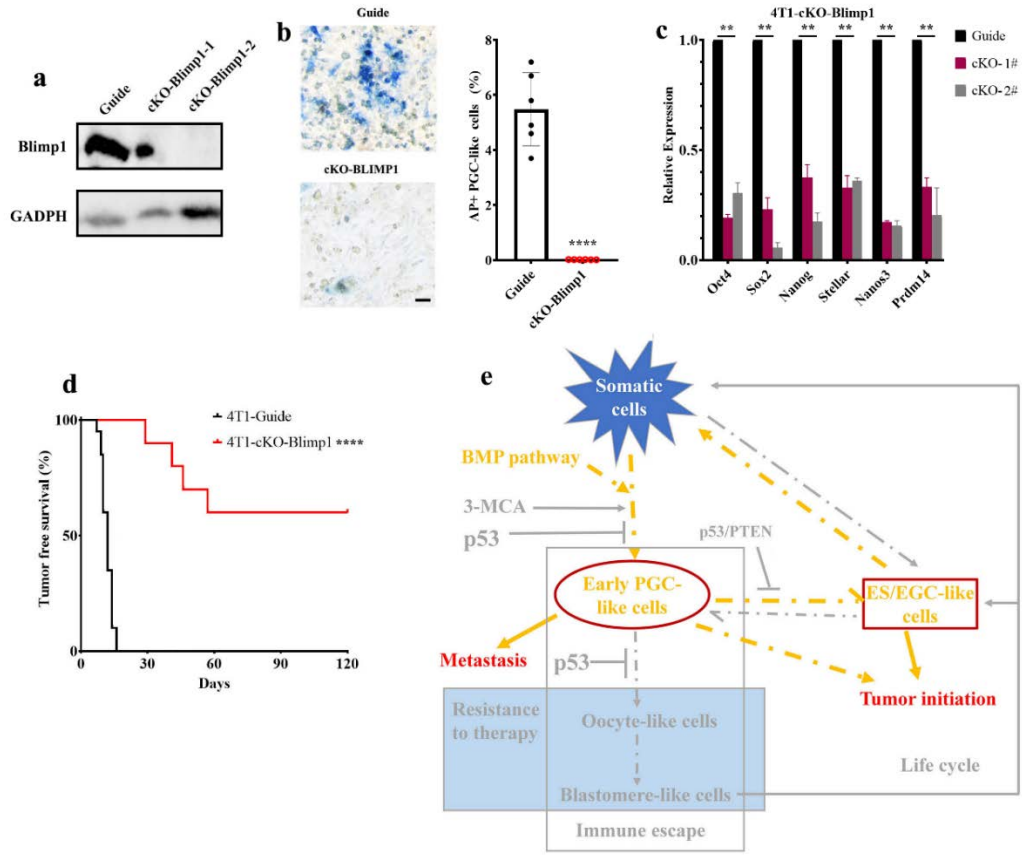

Fig5. Roles of Blimp1 and Smad2 genes in tumour initiation of 4T1 and 168FARN cells. (a) WB results showed that indicated proteins expressed in 4T1 cultures with control or indicated gene knockout. (b) AP staining of 4T1 cultures with control or knockout of indicated genes (left). The percentage of AP^+ PGC-like cells in 4T1 cells with control or indicated gene knockout (right). (c) The mRNA level of indicated genes in 4T1 cultures with control or indicated gene knockout. (d) The survival curve of tumour-free cells showed the difference between 4T1-control and 4T1-KO indicated gene in the ability of tumour initiation. (f) The working model of this finding along with the documents and our previous studies. The yellow showed our current findings of 4T1 cells. The grey lines showed conclusion derived from our previous studies [18, 19] or documents [10, 32]. Scale bar=50 μm (b). **p<0.01, ****p<0.0001.

Discussion

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Our data indicated that acquisition of embryonic/germ cell traits is essential for tumourigenicity. A compelling finding in our study is that tumourigenicity is coupled to the acquisition of PGC-like state through SPLT triggered by the signal pathway involved in PGC specification including Acvr1-Smad1/Smad5-Blimp1 signaling pathway in 4T1 cells, which represent one of aggressive tumour types. In addition, the deletion of any genes related with PGC determination, such as Oct4, Sox2, Nanog, PRDM14, Blimp1, DDX4 and DAZL, inhibited abruptly the formation of PGC-like cells and tumour initiation of 4T1 cells, which provide strong supports for the crucial role of SPLT in tumourigenicity.

Our findings indicated that there might be two distinct ways to gain the tumourigenicity potential from somatic state. The 168FARN cells use one way via reactivation of ES-like state to obtain tumourigenicity without the metastatic capability. Consistent with this finding, ES cells typically exhibit the tumourigenicity but non-invasive [37]. In this case, the PGC-like state is not crucial for tumourigenicity. However, the 4T1 cells utilize another way via ES/PGC-like state from somatic state through the activation of SPLT. In this case, the PGC-like state is critical for tumourigenicity and metastasis [19]. Consistence with this, some human tumours showed strong metastatic feature at the beginning, which is even earlier than the time when clinic diagnosis was made. Thus, targeting SPLT appears to be a promising strategy for combating those tumour types resembling 4T1 cells.

Somatic to germ cell transformation naturally occurs in plants and a few animal phyla such as cnidarians, flatworms and tunicates [38]. It also appears spontaneously in the Caenorhabditis elegans strains with long life-span and inactivation of Rb homolog LIN-35 promotes the transformation[35, 39, 40]. The activation of PGC-like state in tumours can be facilitated by *p53* deficiency[18, 38] possibly representing aggressive stage, consistent with its essential role in metastasis. Combined with our previous studies and literatures[38] our findings indicated that embryonic/PGC-like state might be core phenotypes to drive tumour initiation and that PGC-like properties were also associated with metastatic ability (Fig. 5f). Moreover, the PGC-like tumour cells possibly return to somatic state via the pathways of PGC-EGC like conversion and/or parthenogenetic activation of oocyte-like tumour cells (Fig. 5f). In summary, our findings reveal that tumourigenicity is attributed to the acquisition of PGC-like state through SPLT in 4T1 cells not but merely the activation of germ cell-related genes[24], which may enable to deeper understand the biological nature of tumours through embryonal/gametogenesis-related theory of tumours and provide useful targeted strategies for tackling cancer resembling 4T1 cells.

Materials and methods

Ethics statement

Our animal experiments were carried out in compliance with the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (2020). All animal experiments were approved by with the Institutional Animal Care and Use Committee of Fudan University (approval number: 2019JS-073).

Cell culture

The 168FARN (obtained from Dr. Kounosuke Watabe), 67NR (obtained from Dr. Kounosuke Watabe) and 4T1 (from ATCC cell) cell lines originated from the same breast tumours of BALB/c mice. Transformed bone marrow-derived cells-7 (TBMDCs-7) and TBMDCs-2 were generated from bone-marrow of $p53^{+/+}$ and $p53^{-/-}$ mice respectively and then underwent carcinogenesis by treatment with 3-methyl-cholanthrene (3-MCA) in vitro [19]. For all the cell culture, high-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS; Sigma), 1% L-glutamine and 37 °C with 5% CO2 were used.

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Real-time PCR analysis

RNA was obtained from various cells with Trizol reagent (Invitrogen) according to the manufacturer's protocol followed by reverse transcription to cDNA with a reverse transcription kit (Invitrogen). Real-time PCR of the cDNA was performed with the SYBR Green PCR Master Mix Kit (Applied Biosystems). Primers were shown in our previous study [19] and the Supplementary Table S1.

Alkaline phosphatase staining

Cultures were fixed using 4% paraformaldehyde in PBS for 4 min, washed twice using a Tris-HCl (pH = 8.2) buffer solution and then incubated with AP kit (Vector Laboratory) overnight at room temperature.

DMH2 treatment

4T1 cells were treated with DMSO or DMH2(3 μM, 6 μM or 8 μM) for 24h and 48h respectively and the protein expression was detected with WB. 4T1 cultures (1×10^5 cells) were treated with DMH2 (8 μM) for 12h, 24h and 48h respectively and the mRNA levels at the different time point were detected. After treatment with DMH2 (8 μM) for 48h, the 4T1 cells were incubated in normal medium for 12h, 24h or 48h and the mRNA levels at the different time points were detected. The 4T1 cultures treated with DMSO or DMH2 for 48h were injected into mice (n=5) for tumour development.

Antibody

The primary antibodies used in the study included anti-Oct4 (ab184665, Abcam), anti-Sox2 (MAB2018R-100, R&D), anti-Nanos3 (ab70001, Abcam), anti-Stellar (Invitrogen, PA5-34601), anti-PRDM14 (ab187881, Abcam), anti-DDX4 (ab27591, Abcam), anti-DAZL (NB100-2437, Novus biologicals), anti-Smad2 (ab33875, Abcam), anti-Acvr1 (ab155981, Abcam), anti-Smad1 (ab33902, Abcam), anti-Smad5 (ab92698, Abcam), anti-Smad4 (ab40759, Abcam).

Immunofluorescence

Cultures were incubated in chamber slide for 24h and then fixed with 4% paraformaldehyde and stained with several primary antibodies, including anti-Oct4 (1:400), anti-Stellar (1:200; rabbit; AbCam), anti-DAZL (1:300) or anti-DDX4arom (1:200), washed and then incubated with secondary antibodies conjugated fluorescent dye (Alexa Fluor 488 or 555). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen).

Deletion of genes with CRISPR–Cas9 technology

The CRISPR/Cas9 (Addgene) and mouse sgRNA-RFP (Sigma) plasmids were used to knockout indicated genes while the guide empty vector (Sigma) was used to serve as the control. X-tremeGENE9 DNA transection Reagent (Roche, 20μl) and Opti-MEM medium (Gibco, 500 μl) were mixed for 5 min, incubated with 6 ng of DNA (090, 091 plasmids 3:1) and 6 ng of CRISPR/Cas9 for 25 min at room temperature, and then these mixtures were added to HEK293T cells with 9.5 ml medium. The medium with the viruses in HEK293T cells was collected after cultured for 24h and 48h respectively, filtrated with a 0.45 μm Steri-Flip filter (Millipore), mixed with 20% fresh culture medium containing 7 μg/ml polybrene and then used to transfected 4T1 or 168FARN cells for 4 h. After infected twice with medium with virus and then cultured with normal 10% fresh medium for 48h, the 4T1 or 168FARN cultures were treated with 4 μg/ml blasticidin for 5 days to obtain the 4T1-Cas9 or 168FARN-Cas9 cells. Then transfected indicated sgRNA or guide plasmid to 4T1-Cas9 or 168FARN-Cas9 cells with the same protocol and treated with 5 μg/ml puromycin for 5 days and then isolated single clone with 96-well plates. DNA sequencing and western blot was performed to select the knockout cell clones. The sequencing results of the knockout cells and sgRNA information were provided in Supplementary Table S2.

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Western blotting

Cultures were collected, washed with ice-cold phosphate-buffered saline (PBS), and lysed in 0.5 ml ice-cold radioimmunoprecipitation (RIPA) buffer protease/phosphatase inhibitors for 20 min on ice. Protein was harvested after centrifugation and then boiled in Laemmli buffer for 15 minutes at 4°C. Proteins were resolved by SDS–PAGE and transferred onto a 0.45 mm nitrocellulose membrane (Millipore). Membranes were blocked in 5% milk for 2h, incubated overnight at 4°C with indicated primary antibody, and then incubated with secondary antibody. Bound antibodies were visualized using and then detected with Imaging System (ChemiScope 6000).

Xenograft animal experiments

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care. For comparing the tumourigenicity, the 4T1 (5×10^5), 168FARN (5×10^5), 67NR (5×10^5) cells, 4T1 treatment with DMSO or DMH2 as well as the sorted Stellar$^+$ (100 cells) and Stellar$^-$ (100 cells) from 4T1 cells were subcutaneously grafted into 5 BALB/c mice, respectively. For analyzing the roles of the indicated genes, the 4T1 cells knocked out with Oct4, Sox2, Nanog, PRDM14, DDX4, DAZL, Acvr1, Smad1, Smad5 or Blimp1 with CRISPR-Cas9 technology and the 4T1 control cells were subcutaneously grafted into 10 BALB/c mice, respectively. The time of tumour initiation was recorded. All animal studies according to protocols approved by the Laboratory Animal Committee of Fudan University and handled with care and euthanized humanely during the experiment.

Cell sorting with FACS

The 4T1 cell cultures were collected by trypsinization, incubated with the antibody against Stellar for 1h on ice, stained with secondary antibody conjugated to Alexa Fluor488, and then sorted with the flow cytometry sorter.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 6.0 (San Diego, CA, USA). Kaplan–Meier curves and Unpaired t test with Welch's correction were used to assess overall survival and the difference of gene expression between distinct groups respectively. All experiments were repeated at least three times. $P < 0.05$ were considered as statistically significant.

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Author contributions:

Z. M., Z.F. and A.L. performed the experiments and analysed the data from the Chinese Glioma Genome Atlas (CGGA). C.L. and HK. L designed the experiments, analysed the data and wrote the paper.

Availability of supporting data

All data are available in the main text or in the supplementary materials.

Consent for publication

Not applicable

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Conflict of interest

The authors declare that they have no conflicts of interest.

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