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Forum

Pursuing totipotency: authentic totipotent stem cells in culture

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Totipotent stem cells are transiently occurring *in vivo* cells that can form all cell types of the embryo including placenta, with their *in vitro* counterparts being actively pursued. Subsequently, totipotent-like cells are established with variable robustness and biological relevance. Here, we summarize current progress on capturing these cells in culture.

Totipotency (see Glossary) is the ability of a single cell to form viable embryos including the embryo proper as well as extraembryonic placenta and yolk sac. However, mixed usage of different definitions have caused confusion in the field. Some groups have defined it as the capacity of a group of cells to contribute to both embryonic and extraembryonic tissues. In contrast, a relatively more stringent definition by others refers to the ability of a single cell to contribute to both embryonic and extraembryonic lineages of a developing embryo. By the strictest definition, only zygote and two-cell (2C) stages of developing embryos are genuinely totipotent for their ability to give rise to viable organisms, referred to as *in vivo* canonical totipotency. In contrast, those *in vitro* grown cells with less stringent definitions are referred to as *in vitro* experimental totipotency [1].

Totipotency exists transiently in zygote and 2C embryo stages during early development, which subsequently commit to two distinct lineages; that is, the embryonic cell lineage (**inner cell mass**, ICM) that forms embryo proper and the extraembryonic cell lineage (trophectoderm, TE) that forms placental tissue (Figure 1A). Embryonic stem cells (ESCs) are derived from the ICM and have the capacity to develop all three primary germ layers, a unique feature of **pluripotency**. However, they fall short of the extraembryonic lineage contribution associated with totipotency (Figure 1B, Table 1). The gold-standard approach for generating authentic totipotent cells is somatic cell nuclear transfer (SCNT) technique, pioneered by Sir J. G. Gurdon in 1962 in frogs and culminated in the cloning of Dolly the Sheep by Ian Wilmut in 1996 (Figure 1A). However, SCNT involves transferring a somatic nucleus into an enucleated oocyte and suffers from technical challenges due to its poor efficiency rate and ethical challenge for using oocytes in human studies. Therefore, alternative approaches to capture the *in vitro* experimental totipotency are actively pursued in the field.

In vitro experimental totipotent-like cells and their biological properties

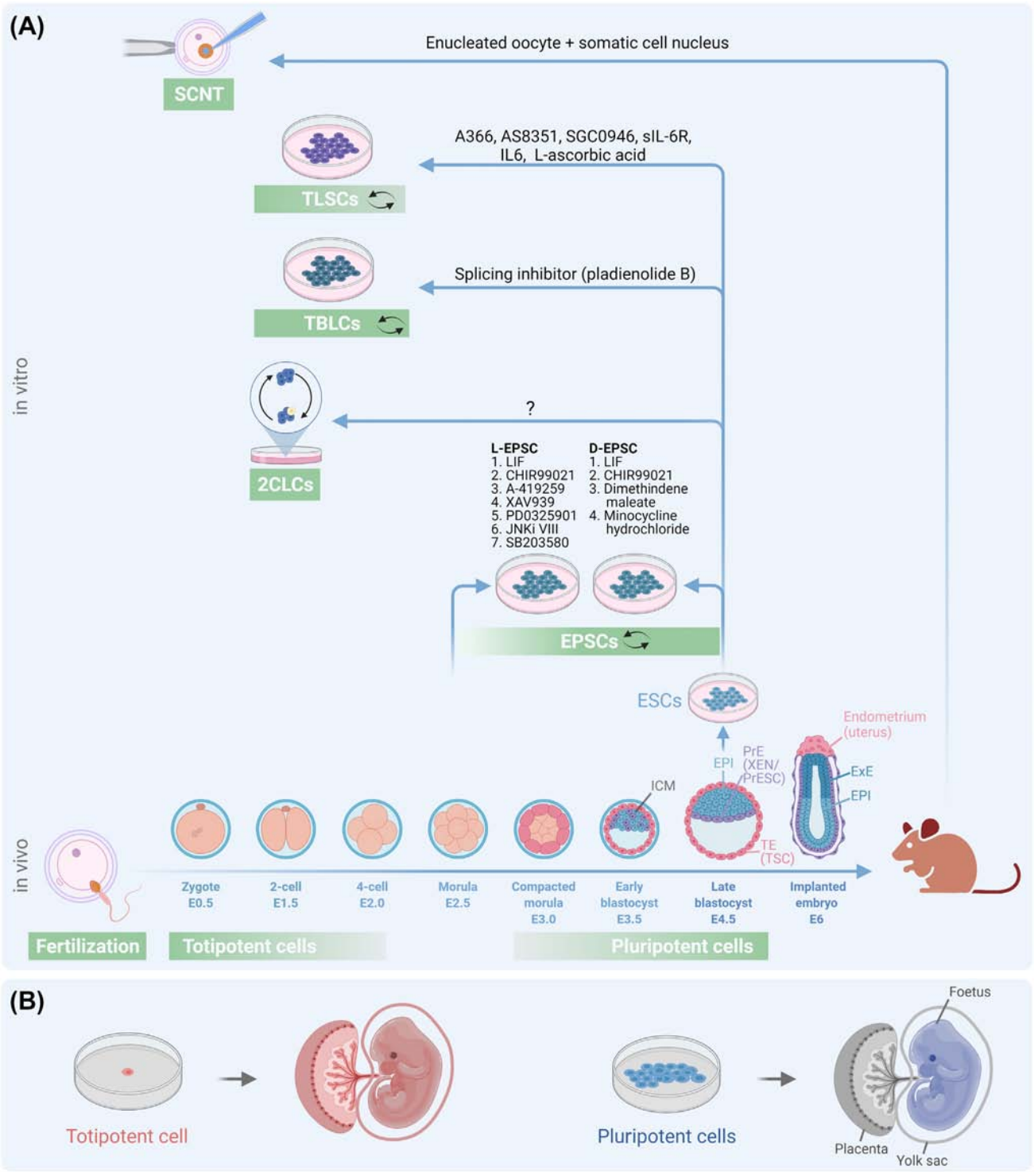
The journey towards capturing totipotent-like cells started with cultured pluripotent cells. In 2012, the Pfaff laboratory identified a rare transient population of **2 cell-like cells** (2CLCs) within mouse ESCs [2] that shared transcriptomic, genomic, and metabolic features with that of totipotent 2C stage embryo (Figure 1A). Transcriptomic profiling during ESC to 2CLC transition showed the emergence of Zscan4^{+} cells, downregulation of pluripotency followed by activation of 2C-specific gene circuitry as intermediate states [3,4]. Functional studies identified *Myc*, *Dnmt1*, *Prc1.6*, and *Ep400-Tip600* as barriers [3,4] and *Dux*, *Gata2*, *Eif3h*, *Dppa2/4*, *Nelfa*, and *Atr* as facilitators for 2CLC emergence [5]. Mechanistically, Dux activates 2C-related genes by binding directly or indirectly by binding and activating the *miR-344* cluster, which post-transcriptionally silences *Zmym2* and *Lsd1* resulting in de-repression of *MERVL* and other 2C-specific genes [6].

Glossary

2-cell like cells: transiently appearing cells within ESC culture where ESCs dynamically enter and exit a 2C-like state with 1–5% cells at equilibrium at a given time.
Blastocyst: the blastocyst of the mammalian embryo follows morula (solid ball of cells) stage by forming a hollow ball of cells with a fluid cavity (blastocyst), consisting of ICM and an outer layer trophoblast.
Epiblast: blastocyst cells that form almost all fetal tissues and the extraembryonic mesoderm.
Expanded/extended potential stem cells: *in vitro* totipotent-like cells that are derived from 8-cell stages or ESCs using small molecule inhibitors.
Extraembryonic endoderm stem cells: XEN cells are derived from the PrE and act as a useful model of PrE and serve for differentiation into visceral and parietal endoderm.
Inner cell mass: a blastocyst cell population that contains the precursors of EPI and PrE lineages.
Pluripotency: ability of a single cell to give rise to all cell types of a body but not the extraembryonic or placenta cells.
Totipotency: ability of a single cell to give rise to all cell types in a body plus extraembryonic or placenta cells.
Totipotent blastomere-like cells: *in vitro* totipotent-like cells derived from mESCs using a splicing inhibitor and resemble more closely to 2C and 4C stages of early embryonic development.
Totipotent-like stem cells: derived from mESCs using chemical reprogramming that show molecular and functional features similar to 2C embryos.
Trophectoderm: outer layer of the blastocyst, which will form fetal portion of placenta and outer layer of parietal yolk sac.
Trophoblast stem cells: precursor cells of the placenta that contribute specifically to the trophoblastic component of placenta and parietal yolk sac.

Owing to their contribution to embryonic and extraembryonic lineages, 2CLCs are proposed to have an expanded cell fate potential [2,7]. However, stable 2CLCs *in vitro* has not been captured in culture, and their totipotency status has not been rigorously tested according to current standards (Table 1; Box 1).

Using small molecules, Liu and Deng laboratories generated **expanded/extended potential stem cells** (EPSCs) from mouse eight-cell embryos, mouse ESCs (mESCs), human ESCs (hESCs), and human induced pluripotent stem cells (hiPSCs) [8,9] (Figure 1A). EPSCs show totipotency-associated marker gene expression and



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possess the ability to contribute to both embryonic and extraembryonic tissues, including trophoblast lineages in chimera generation (Table 1). While ESCs need trophoblast stem cells (TSCs) and/or extraembryonic endoderm (XEN) cells to form into a blastoid-like structure, even a single EPSC could do so [10]. Such potential fits the *in vitro* experimental totipotency definition. However, contrary to previous claims, single-cell transcriptomic profile comparison showed Liu-EPSCs correlated more with E4.5 or parental ESCs and Deng-EPSCs with E5.5 epiblast or EpiSCs rather than earlier embryonic states [11]. EPSCs also failed to facilitate TSC reprogramming. Although EPSCs contributed to both **epiblast** (EPI) and **trophectoderm** (TE) in chimera aggregation experiments, a careful examination of cells contributing to TE showed expression of pluripotency factors rather than TE or trophoblast lineage markers [11], suggesting the *in vitro* experimental totipotency of EPSCs could be limited (Table 1). Although EPSCs also underperformed in blastoid-forming assays, a small population could nonetheless contribute to TE or extraembryonic endoderm (ExE)-like cells. This brings up a contrasting yet intriguing point that only the forced positional localization of donor EPSCs to surface in blastoid-forming assays, but not freely localized donor EPSCs to the host embryo in chimera aggregation experiments, may induce TE differentiation. These observations suggest that not all but only a tiny EPSC population might have expanded potential under specific experimental conditions. Thus, future totipotency capture and assessment should focus on the differentiation of proper cell types and their positions in the embryo to satisfy strict standards.

In the race to obtain *in vitro* totipotent cells, the Du laboratory has made fascinating claims of generating **totipotent blastomere-like cells** (TBLCs) from mESCs (Figure 1A) using a splicing inhibitor [12]. TBLCs show epigenomic and transcriptomic profiles similar to 2C and four-cell blastomeres but distinct from either EPSCs or 2CLCs and have robust bidirectional embryonic and extraembryonic differentiation potential (Table 1). However, in the current culture conditions, these cells suffer from a low proliferation rate, and the molecular basis for self-renewal of TBLC warrant further experiments. Nonetheless, this study uncovers another way to capture and maintain totipotent-like cells *in vitro*.

More recently, **totipotent-like stem cells** (TLSCs) were derived from mESCs using chemical reprogramming (Figure 1) that showed remarkably similar features to 2C embryos than any other totipotent-like cells derived so far [13]. TLSCs share 2C-stage transcriptomic, chromatin and broad-H3K4me3 profiles and contribute to both embryonic and extraembryonic tissues in *in vivo* chimera and *in vitro* blastoid-formation assays following the stringent criteria for totipotency assessment (Table 1). Although TLSCs present a suitable model for understanding establishment, maintenance, and exit of totipotency, they are heterogeneous cells in culture and, like all other totipotent-like cells established thus far, the blastoids derived from TLSCs are incapable of developing into an entire organism.

The last decade has seen a surge of *in vitro* cultured stem-cell-based embryo models using human EPSCs, iPSCs and especially human naïve ESCs [14]. Compared to conventional human primed ESCs, naïve hESCs markedly display a broader developmental potential to form **blastocyst**-like structure and contribute to both EPI and TE [15]. Naïve ESC-based blastoids, generated with triple inhibition of Hippo, transforming growth factor (TGF)-β, and extracellular signal-regulated kinase (ERK) pathways, showed better similarity to their *in vivo* counterparts including implantation capability using hormone stimulated 2D open-faced endometrial layer (OFEL) system *in vitro* [15]. However, these human blastoids do not represent early preimplantation (zygote to morula) stages thus making *in vitro* derived totipotent-like cells a more desirable system to capture early and late stages of embryogenesis.

The above-mentioned *in vitro* derived totipotent-like cells were all shown to have varying degrees of expanded lineage differentiation potential compared to ESCs. Yet they are molecularly and functionally distinct (Figure 1A; Table 1). The striking differences in the acquiring approaches and molecular features of these cells could suggest that

Figure 1. Overview of mouse early development and current status of totipotent-like cells cultured *in vitro*. (A) Mouse development begins with fertilization followed by the formation of a zygote (1-cell) and blastomere of a 2-cell (2C) embryo; both of which are totipotent. The zygotic genome is activated at the 2C stage in the mouse. The inner cell mass (ICM) cells in the blastocyst are pluripotent. The blastocyst has three distinct cell lineages: EPI, epiblast; PrE, primitive endoderm; and TE, trophectoderm. EPI gives rise to all cells of the organism; TE forms placenta; and PrE forms yolk sac. Embryonic stem cells (ESCs), **extraembryonic endoderm (XEN) stem cells** or primitive endoderm stem cells (PrESC) cells (see Ref [1] in the supplemental information online), **trophoblast stem cells** (TSCs) have been established from EPI, PrE and TE respectively, by isolating cells from mouse blastocysts. Differentiated cells can be reprogrammed to totipotent cells through somatic cell nuclear transfer (SCNT). ESCs have been reprogrammed to totipotent blastomere-like cells (TBLCs) via spliceosome suppression [12], to expanded/extended potential stem cells (EPSCs [8,9]) and totipotent-like stem cells (TLSCs [13]) via small molecule inductions. In contrast, **2 cell-like cells** (2CLCs) are transiently appearing cells within ESC culture where ESCs dynamically enter and exit a 2CLC-like state with 1–5% cells at equilibrium at a given time [2,6]. Key chemical inhibitors used for *in vitro* derivation of indicated TLSCs are mentioned. Green rectangles roughly represent the molecular features matching with early mouse development stages. Two small, curved arrows represent self-renewal *in vitro*. E0.5 to E6 represent embryonic day numbers. ExE: extraembryonic endoderm. (B) Schematic depiction of totipotent (red) and pluripotent (blue) cells and their contributions to both embryonic and extraembryonic tissues or only to embryonic tissues, respectively. Drawn with BioRender.com.

Table 1. Molecular features of pluripotent and *in vitro* experimental totipotent-like cells

Features	mESCs	hESCs (naïve)	2CLCs [2]	EPSCs [8,9]	TBLCs [12]	TLSCs [13]
Embryonic tissue contribution	Yes	Yes	Yes	Yes	Yes	Yes
Extra embryonic contribution to TE						
(i) Chimera assays (*in vivo*)	No	Ethically challenging	Yes[a]	Yes[c]	Yes	Yes
(ii) Blastoid-formation assay (*in vitro*)	No	Yes	Yes[b]	Yes[c]	Yes	Yes
(iii) TSC reprogramming	No	Yes	Not tested	Yes[c]	Not tested	Not tested
Transcriptomic profiles						
Global comparison with mouse early embryonic states (single-cell RNA-seq)	Similar to preimplantation epiblast stage	Similar to preimplantation epiblast stage	Not compared with *in vivo* totipotent cells	Similar to 4C and 8C stages[c]	Similar to 2C and 4C stages	Similar to 2C and 4C stages
Totipotency or 2C markers	No	No	High	No	High	High
Pluripotency markers	High	High	Low	High	Low	Low
MERVL	Low	Show HERV expression	High	No	High	High
Genomic landscape						
Chromatin mobility	Low	comparable	High	Not tested	Not tested	Not tested
Chromatin accessibility	Low	comparable	High	Not tested	High; 8C and ICM-specific open ATAC-seq peaks	High, 2C and 4C specific open chromatin
DNA methylome	High	comparable	Low	Intermediate/ dynamic[d]; higher level of 5hmC	Low	Not tested
In vitro culture						
Self-renewal	Yes	Yes	Transient	Yes	Yes	Yes

Here high/low and yes/no are based on comparison with mESCs.
[a]Not tested with stringency [7].
[b]Done using EpiSC-derived iBLCs (see Ref [2] in the supplemental information online), which are molecularly similar to 2CLCs; also not done with single 2CLC.
[c]Challenged by [11].
[d]Varying levels of 5mC were found between mESCs cultured in different media and EPSCs generated from mESCs of different genetic backgrounds [8].

there exist alternate routes to reach an authentic totipotent state or alternative totipotent states.

Ongoing capture of *in vitro* experimental totipotency

Lessons from *in vivo* embryogenesis and ground-breaking discovery for induction and maintenance of iPSCs should provide a guiding torchlight for establishing authentic totipotent cells *in vitro*. The forced ectopic expression of transcription factors (TFs) could similarly be used to induce totipotency in pluripotent and/or somatic cells following a rational selection of factors, involved in both induction and maintenance of totipotency. For example, although not essential for embryonic development (see Ref [3] in the supplemental information online), the Dux protein family gene *Dux* (human homolog *DUX4*) could be a potential candidate as its overexpression in ESCs can partially activate ZGA and cause chromatin changes similar to 2C stage embryos see Refs [4, 5] in the supplemental information online).

Extracellular signaling is also critical in regulating cell fates by circumventing limitations of low efficiency, slow kinetics, viral vector dependence, and multiple TF requirement. Additionally, small molecules are rapid, safe, dose-dependent, more controllable, and have the potential for high-throughput screening and therapeutic interventions. High bone morphogenetic protein 4 and low Activin/Nodal signaling are speculated to be the reason for expanded potential of EPSCs [10,11]. Similarly, using chemical inhibitors, triple inhibition of Hippo, TGF-β, and ERK pathways enhanced human blastoid generation [15]. Thus, improving chemically defined culture conditions will accelerate totipotency reprogramming and facilitate establishing bona fide *in vitro* experimental totipotency.

The success of all above-mentioned strategies largely relies on a robust reporter system. The most commonly used reporter system so far is MERVL–Zscan4 dual reporter for mouse 2CLCs [2,4], yet a robust reporter for human systems is currently lacking and thus a TBLC- or TLSC-like

Box 1. Tests for assessing the authenticity of *in vitro* captured totipotent-like cells

Since there is some confusion in defining the true nature of *in vitro* derived totipotent-like cells (Figure 1A), gold standard criteria for their assessment are crucial. Currently, totipotency is assessed using the following standards. (i) Transcriptomic profiling, especially using single-cell RNA-seq, determines the appropriate cellular state as well as heterogeneity relative to *in vivo* totipotent cells. (ii) Monitoring cellular epigenome during long-term cultures (not done for EPSCs, TBLCs and TLSCs, Figure 1A and Table 1). (iii) TSC differentiation tests the differentiation capacity of totipotent-like cells to trophoblast lineage by switching to TSC culture conditions (only done using EPSCs, Figure 1A, and Table 1). (iv) Assessment of differentiation potential for three embryonic germ layers, TE, PrE, and their derivatives *in vitro*; and chimera formation between donor cells and host embryos followed by lineage contribution analysis at various developmental stages (preimplantation E4.5 and postimplantation E6.5/E12.5) for their *in vivo* contribution to all lineages including trophoblast lineage. Chimera generation is a suitable test to assess pluripotency but with a caveat for totipotency since the host cell stage has already passed the totipotency stage. Therefore, the paracrine signaling donor cells receive in the host environment is not the same as they would have otherwise received in totipotent host stage. (v) The most stringent or gold standard test to date is blastoid-forming assay when a single cell type (even more stringent if only a single cell is used) can form the entire blastocyst and eventually form a live conceptus or entire fertile organism. This assay helps determine appropriate cell types generated and their spatial position in 3D blastoids, suggesting that localization is equally important as differentiation potential when assessing totipotency. However, such an assay could not be implemented for human studies because of ethical reasons, thus for preimplantation morula aggregation and for postimplantation interspecies chimera generation techniques are often conducted.

derivation approach has not been tested for hESCs. An urgent and unmet need is to identify and develop an ideal platform and authentic totipotent reporter for investigating the individual contribution of medium supplements and reprogramming factors that can directly convert somatic and pluripotent cells to acquire *in vitro* experimental totipotency.

Here, we have summarized the current status of existing totipotent-like cells and the technical challenges in generating authentic totipotent cells *in vitro*. The advent of multidisciplinary high-throughput approaches (e.g., genome-wide CRISPR and small-molecule screening) combined with computational (e.g., totipotency reprogramming time-course single-cells genomics data analysis) and experimental methods (e.g., *in vivo* contribution to extraembryonic tissues in chimera and *in vitro* single-cell-based blastoid formation) should enable us to expand our understanding of totipotency and thus empower us to capture and exploit these totipotent-like cells in developmental biology, fertility, designing new contraceptives, generating organs, and regenerative medicine.

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Declaration of interests

No interests are declared.

Supplemental information

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