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PRIMER

From pluripotency to totipotency: an experimentalist's guide to cellular potency

Alba Redó Riveiro and Joshua Mark Brickman*

ABSTRACT

Embryonic stem cells (ESCs) are derived from the pre-implantation mammalian blastocyst. At this point in time, the newly formed embryo is concerned with the generation and expansion of both the embryonic lineages required to build the embryo and the extra-embryonic lineages that support development. When used in grafting experiments, embryonic cells from early developmental stages can contribute to both embryonic and extra-embryonic lineages, but it is generally accepted that ESCs can give rise to only embryonic lineages. As a result, they are referred to as pluripotent, rather than totipotent. Here, we consider the experimental potential of various ESC populations and a number of recently identified *in vitro* culture systems producing states beyond pluripotency and reminiscent of those observed during pre-implantation development. We also consider the nature of totipotency and the extent to which cell populations in these culture systems exhibit this property.

KEY WORDS: Developmental biology, Pluripotency, Totipotency

Introduction

Embryonic development proceeds from a single fertilized egg to generate all lineages that make up the future organism. In mammals, the initial rounds of cell division result in the expansion of a pool of equipotent cells (termed blastomeres) that represent the founder cells of all prospective cell types. In mice, humans and all eutherian mammals, these initial stages of development deal with the specification of both the embryo proper and the supporting structures required for placental development, the embryonic region of the placenta, as well as the yolk sac. Based on a long history of experimental manipulation, it has been demonstrated that early embryonic cells start with the capacity to make all lineages. However, this capacity – the potency of cells – becomes restricted as development proceeds.

For the purpose of this Primer, we wish to first explicitly define and describe terms related to cell potency in early development. Although assays for cellular potency have been undertaken using a range of mammalian species, the majority of experiments have been carried out in mouse, and this is where we focus. The strictest and most widely used definition of totipotency is the ability of one cell to give rise to a fully functional organism. The ultimate test for totipotency is to add one cell into an empty zona pellucida (the glycoprotein layer surrounding a developing embryo) and transfer it to the uterus of a pseudo-pregnant mouse to resume development. In this test, a totipotent cell is able to generate the entire conceptus or fetus, including a functional germ line, as well as the supporting extra-embryonic tissues required for its development. By this canonical definition, in mice, only the zygote and the blastomeres of the 2-cell (2C) stage embryo are truly totipotent cells. Pluripotency, by contrast, refers to the ability of a cell to develop into the three germ layers (ectoderm, mesoderm and endoderm), in addition to the germ line, and therefore into all lineages contained within the adult body but not within the extra-embryonic lineages. Although contribution to the germ line is not an absolute requirement for all definitions of pluripotency (e.g. for human cells), it was historically viewed as the gold standard for pluripotency. A typical test for pluripotency involves injecting one or several cells into an early embryo, typically a blastocyst or morula. In this test, a pluripotent cell such as an ESC is able to contribute to the embryonic tissues of the resulting chimera, whereas the extra-embryonic tissues are derived entirely from the host. In some instances, injection experiments can produce chimeric mice derived entirely from donor cells, but this is not a requirement for pluripotency.

In the past 10 years, attempts have been made to push the boundaries of pluripotent cell culture in order to generate pluripotent cells that exhibit some levels of totipotency, also referred to as 'extended' or 'expanded' potency. Some of these cells harbor the capacity to give rise to both embryonic and extra-embryonic lineages. Therefore, to provide a definition of totipotency consistent with that historically used for pluripotency, we use the term 'experimental totipotency' to define this capacity of a cell to simultaneously contribute to embryonic and extra-embryonic tissues in chimera experiments. As the terms extended or expanded potency do not explicitly define the capacity to generate all lineages, i.e. a greater potency than pluripotency, we believe the term experimental totipotency is more accurate. By analogy to pluripotency, which is the capacity to contribute to but not create an entire embryo, experimental totipotency – unlike canonical totipotency described above – does not require the cells to be able to form a full organism on their own.

In recent years, experimental totipotency has been demonstrated for a number of *in vitro* cell types. However, even where it is proposed to be demonstrated, considerable controversy exists. Here, we try to place these experiments in context, contrasting the precise nature of these claims without making a specific judgment on the validity of these results. We discuss the evidence for experimental totipotency, the different phenotypes manifested in cells that exhibit this experimental property and the culture systems that enable their propagation. We also focus on the historical definitions of embryonic potency and discuss how these relate to the properties of pluripotent stem cell models and their competence to differentiate towards extra-embryonic lineages. We contrast traditional pluripotent cell culture conditions to more recent innovations reported to enhance experimental totipotency and discuss the populations supported in these cultures. Finally, we also provide a

Novo Nordisk Foundation Center for Stem Cell Biology (DanStem), University of Copenhagen, Blegdamsvej 3B, DK-2200 Copenhagen N, Denmark.

*Author for correspondence (joshua.brickman@sund.ku.dk)

A.R.R., 0000-0002-7015-2486; J.M.B., 0000-0003-1580-7491

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comprehensive list (Table S1) of experimental approaches that have been used to probe cellular competence and the extent to which these assays have been validated quantitatively. We hope this will help the reader formulate their own view about the capacity of *in vitro* cell cultures to recapitulate embryonic development.

An overview of pre-implantation development and its gene regulatory networks

Following fertilization, the totipotent zygote initiates development, undergoing a series of symmetric cell divisions known as cleavages, leading to the formation of the morula (Fig. 1). Cleavages have a short G1 and long S phase (Gamow and Prescott, 1970), and during this period there is no cellular growth or increase in cell mass; therefore, the embryo maintains the same size as the original zygote, but with a larger number of smaller blastomeres.

After the first few rounds of cell division, at embryonic day (E) 3.0 in mouse, the first lineage specification event occurs, leading to the segregation of the trophoblast (or trophectoderm, TE) and the inner cell mass (ICM). The TE will develop into the future placenta, while the ICM will undergo another partitioning at E4.5 to generate the epiblast (Epi) and primitive endoderm (PrE). The Epi then gives rise to the embryo proper including the germ line, while the PrE further differentiates into the extra-embryonic visceral endoderm and the parietal endoderm. The transcription factors (TFs) that are later expressed in the TE, Epi and PrE (e.g. CDX2, NANOG, SOX2, KLF4 and GATA6) are co-expressed in early blastomeres (Dietrich and Hiiragi, 2007; Guo et al., 2010; Morgani and Brickman, 2015). As development progresses, the expression of lineage-specific TFs gradually becomes mutually exclusive as cells become increasingly specified.

The initial specification of ICM and TE cells occurs as a result of polarization of the outer cells and is dependent on the Hippo and Notch signaling pathways (Nishioka et al., 2009; Rayon et al., 2014). Indeed, a large body of work has focused on the role of Hippo in the polarized outer cells, where YAP1 translocates to the nucleus to activate, via TEAD4, TE-specific transcriptional programs and factors, including TE determinants such as Cdx2 and Eomes (Cockburn et al., 2013; Hirate et al., 2013; Nishioka et al., 2008, 2009). Although cell-cell contact is required for ICM specification, experiments using isolated blastomeres suggest TE specification can occur by default (Lorthongpanich et al., 2012). By E3.0, the TE and ICM lineages become distinct and are supported by the reciprocal inhibitory relationship between CDX2 expression in the TE and OCT4 in the ICM (Niwa et al., 2005).

As the morula increases in size to 32 cells, it cavitates to form a blastocyst and the ICM becomes localized to one side of the blastocoel cavity (Fig. 1). The ICM cells then undergo a second lineage segregation event, generating the Epi and the PrE. Initially, cells of the mouse ICM co-express TFs specific to the Epi and PrE, such as NANOG and GATA6, respectively (Plusa et al., 2008; Morgani and Brickman, 2015; Ohnishi et al., 2014; Saiz et al., 2016). However, at E3.5, the expression of these two markers becomes mutually exclusive, generating a salt-and-pepper pattern of PrE and Epi progenitors throughout the ICM that subsequently resolves into two spatially segregated cell layers (Chazaud et al., 2006; Plusa et al., 2008; Xenopoulos et al., 2015). By E4.5, the

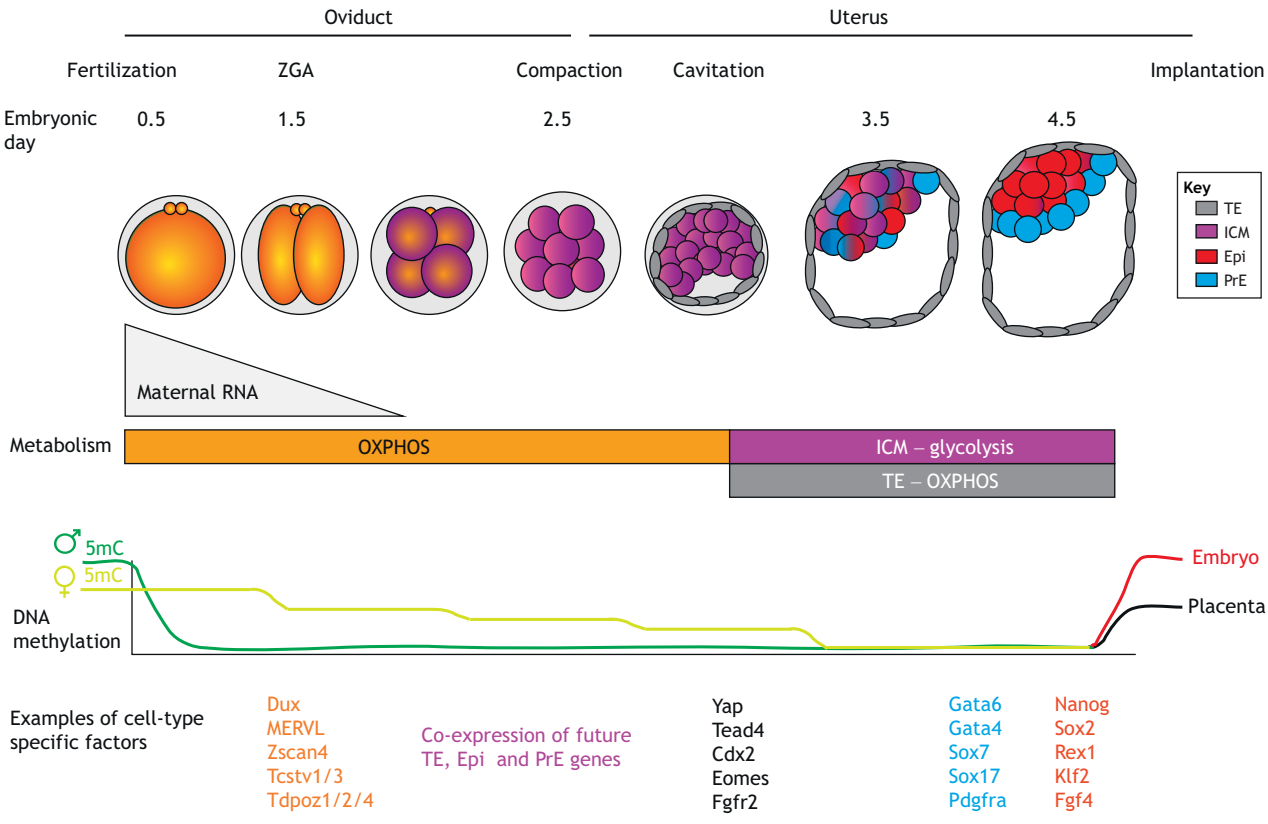

Fig. 1. Schematic illustration of mouse preimplantation embryo development. Summary of early mouse development highlighting the expression of specific markers and cell states. Changes in metabolism, DNA methylation and the levels of maternal RNAs are also highlighted. Epi, epiblast; ICM, inner cell mass; PrE, primitive endoderm; TE, trophectoderm; ZGA, zygotic genome activation.

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lineages are completely separated and the PrE is positioned between the Epi and the blastocoel cavity.

The resolution of GATA6- and NANOG-positive progenitor populations is regulated by FGF/MAPK signaling. Treatment of cultured blastocysts with FGF4 blocks Epi formation in mouse blastocysts, whereas sustained inhibition of the FGF/ERK pathway promotes Epi differentiation at the expense of the PrE (Yamanaka et al., 2010). FGF4 mutant embryos are unable to generate PrE, although the initial expression of GATA6 in unsegregated ICM cells is unaffected (Kang et al., 2013; Krawchuk et al., 2013). Modulating the level of ERK activity downstream of FGF4 thus determines Epi or PrE identity, and this is partially achieved by the combination of two FGF receptors (Kang et al., 2017; Molotkov et al., 2017). The segregation of these lineages is also driven via gene regulatory networks and paracrine signaling, as Epi-biased cells (NANOG high expressers) secrete FGF4, which signals to neighboring cells, inducing them to adopt a PrE fate (Bessonnard et al., 2014; Schrode et al., 2014; Saiz et al., 2016; Frankenberg et al., 2011). The mutually antagonistic relationship between GATA6 and NANOG also reinforces this lineage segregation event (Singh et al., 2007; Hamilton and Brickman, 2014; Mitsui et al., 2003; Bessonnard et al., 2014; Frankenberg et al., 2011; Schrode et al., 2014).

During pre-implantation development, the embryo floats freely in the oviduct (E0-2) and then in the uterus (E2.5-E5). At these stages, there is minimal exchange of water and small molecules through diffusion between the embryo and its environment (Kaneko, 2016). As the embryo has no other external energy source, from fertilization to morula stages, it produces energy through oxidative phosphorylation (OXPHOS), exploiting free diffusing monocarboxylates, such as pyruvate and lactate (Brown and Whittingham, 1991; Leese, 2012). Following blastocyst formation, energy demands increase and the embryo switches its source of energy to glucose, with the ICM shifting its mode of production to glycolysis; in the TE, by contrast, cells continue using OXPHOS to generate ATP (Houghton, 2006; Shyh-Chang et al., 2013) (Fig. 1). Although no definitive link between the mode of energy production and totipotency has been established, there is a correlation between the shift away from OXPHOS and the progressive restriction of potency (Kaneko, 2016; Shyh-Chang et al., 2013).

At E4.5, the mature mouse blastocyst hatches from the zona pellucida and implants into the uterine endometrium, initiating decidualization (a process that transforms the endometrium in preparation for pregnancy). Gastrulation then follows at E6.5, during which time embryonic differentiation begins with the specification of the three germ layers and the segregation of the germ line.

Totipotency versus experimental totipotency

ESCs are immortal cell lines isolated via *ex vivo* expansion of the ICM. They can be cultured in a variety of conditions and, as mentioned above, are referred to as being pluripotent; they are able to differentiate into embryonic, but not extra-embryonic, lineages. They also maintain this property through successive rounds of self-renewing cell divisions. It is this definition of pluripotency in ESCs that current questions of what encompasses totipotency are generally framed. Below, we consider the historical context in which totipotency was initially described.

Based on long history of embryology, the early mammalian embryo has been shown to be extremely plastic, with cells readily adapting to changes in cell fate following experimental manipulation, including the bisection or aggregation of embryos (Tarkowski, 1961; Dietrich and Hiiragi, 2007; Suwińska et al., 2008; Grabarek et al., 2012; Saiz et al., 2016; Korotkevich et al., 2017; Nissen et al., 2017). This suggests that early embryonic cells retain potency for multi-lineage differentiation. Extensive embryo manipulation experiments in mammalian embryos have been used to define the potency of individual blastomeres at different stages of development. Tarkowski and others demonstrated that one of the two blastomeres in the 2C mouse embryo can be destroyed and the remaining blastomere is able to give rise to an adult fertile mouse, proving the totipotent nature of a single blastomere of the 2C embryo (Tarkowski, 1959; Papaioannou et al., 1989). Although asymmetries in mRNA distribution that correlate with the capacity to generate intact embryos have been detected, suggesting that both 2C blastomeres are not equipotent, a significant proportion of embryos (27% in these particular studies) contain two canonically totipotent blastomeres (Casser et al., 2017; Casser et al., 2019). This contrast with attempts to generate viable embryos from individual blastomeres from four- and eight-cell embryos; when these are injected into empty zona pellucidae and transferred to pseudo-pregnant mothers, viable embryos cannot be formed. All blastomeres were able to induce the formation of deciduae that contain a few trophoblast giant cells, but only one retarded embryo was formed (Rossant, 1976). As a result, the individual cells of the 2C mouse embryo and the zygote appear uniquely able to generate an entire animal and thus are considered to be totipotent.

Although post-2C stage mouse blastomeres are unable to generate an intact animal on their own, they can generate an entire mouse when provided with support in the form of tetraploid cells (Tarkowski et al., 1977) or additional embryonic material (Kelly, 1977). Under these circumstances, Tarkowski also proved that single blastomeres from the 16-cell embryo retain the capacity to give rise to all embryonic and extra-embryonic lineages, in addition to forming fertile adult mice, regardless of the position of the blastomere (inner or outer) within the embryo. Twins, triplets and quadruplets could also be generated from the same original morula using the tetraploid assay with single blastomeres. However, from 32-cell stage embryos, only outer blastomeres were able to produce all three lineages (TE, PrE and EPI) and rarely could they give rise to an intact embryo (Tarkowski et al., 2010). This restriction in ICM potency appears progressive and may also be influenced by experimental context, as isolated ICM cells from early blastocysts are able to contribute to the trophoblast in morula aggregation, but this property is lost when the ICM expands to more than 16-19 cells (Rossant and Lis, 1979a,b). The capacity of isolated ICM cells to produce TE is also supported by a number of *ex vivo* differentiation experiments (Rossant and Lis, 1979a; Rossant and Lis, 1979b; Suwińska et al., 2008; Grabarek et al., 2012). Although the capacity to generate entire embryos is lost by the 32-cell stage, the capacity of individual cells to differentiate into all cell types is retained in specific sub-populations. Thus, certain ICM cells maintain their plasticity until the late blastocyst stage, with PrE precursors retaining greater plasticity than committed PrE or Epi cells. By E4.5, when the lineages are physically separated, both the PrE and Epi become completely lineage restricted (Grabarek et al., 2012).

Although there has been some discussion regarding the definition of totipotency (Baker and Pera, 2018; Boiani et al., 2019; Condic, 2014; Morgani and Brickman, 2014), the notion that ESCs are pluripotent would suggest that the experimental definitions of potency should be based on the lineages colonized by the descendants of a single cell. While the zygote and cells from the 2C stage embryo retain the unique capacity to form an entire organism on their own, individual cells from the four-cell embryo up to the early ICM are also highly plastic. As we have highlighted

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above, these cells are able to contribute to all three lineages and are even capable of generating a full mouse when aided by tetraploid-supporting blastomeres. We refer to the property of these cells to generate all lineages as 'experimental totipotency', distinguishing it from canonical totipotency.

Pluripotency and pluripotency factors

Self-renewing cell culture models exist for all three preimplantation embryonic lineages, as well as for varying stages of their differentiation. Indeed, the *ex vivo* expansion of cells from the blastocyst in different conditions can give rise to ESCs (Evans and Kaufman, 1981; Martin, 1981), trophoblast stem cells (TSCs) (Tanaka et al., 1998) and extraembryonic endoderm (XEN) cells (Kunath et al., 2005; Niakan et al., 2013).

ESCs were originally isolated from ICM of the blastocyst and transcriptionally resemble the ICM and Epi (Boroviak et al., 2014; Hackett and Surani, 2014; Martin Gonzalez et al., 2016). Later stage epiblast stem cells (EpiSCs) can also be derived from the post-implantation Epi (E5.5-6.5) (Brons et al., 2007; Tesar et al., 2007). Based on this distinction between different stages of epiblast development, the original ICM-derived cells are referred to as 'naïve' pluripotent cells, whereas cells corresponding to later stages of epiblast specification are termed 'primed' pluripotent cells. Conventional human ESCs (hESCs) are generally thought to resemble a primed stage of pluripotency, although a number of naïve ESC cultures have recently been defined for hESCs (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014). Likewise, naïve extra-embryonic endoderm (nEnd) cells can be generated from ESCs; these cells resemble the early PrE when compared with XEN cells, which exhibit a phenotype reminiscent of the parietal endoderm – a PrE derivative (Anderson et al., 2017).

Pluripotency is a functional definition and can be assessed experimentally via three different approaches: by *in vitro* differentiation to the three germ layers, by *in vivo* differentiation based on the capacity to form teratomas when grafted to ectopic sites on adult mice (Evans and Kaufman, 1981), and by *in vivo* contribution to chimeric mice upon embryo injection or aggregation (Gardner, 1968; Rossant, 1976; Bradley et al., 1984; Beddington and Robertson, 1989; Nagy et al., 1990; Wood et al., 1993; Tam and Rossant, 2003; Martin Gonzalez et al., 2016).

Although pluripotency is a state that exists naturally within the blastocyst, and one that is captured in ESCs, it can also be induced via the extrinsic expression of a specific pool of TFs that are referred to as the core pluripotency network. Championing these are the Yamanaka factors OCT4, SOX2, KLF4 and MYC (OSKM), which have been shown to induce pluripotency when overexpressed in somatic cells, generating induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). iPSCs share gene expression profiles with and behave similarly to ESCs (Okita et al., 2007; Takahashi and Yamanaka, 2006). In addition to the above members of the pluripotency network, a number of additional TFs are also known to support or stabilize pluripotency. These include NANOG, PRDM14, ESRRB, TFCP2L1, DPPA3/STELLA, KLF2, KLF5 and TBX3 (Ivanova et al., 2006; Hall et al., 2009; Niwa et al., 2009; Silva et al., 2009; Festuccia et al., 2012; Martello et al., 2013; Yamaji et al., 2013; Russell et al., 2015). These factors support their own expression, as well as the expression of other core pluripotency genes in a concerted feedforward network. This network is complex and heavily buffered with feedforward loops that reinforce the expression of the overall network.

The dynamics of the pluripotency network can be modulated by extrinsic regulators contained in the media in which cells are cultured. Mouse ESCs were originally cultured on feeder cells in the presence of serum (Evans and Kaufman, 1981). Feeders were found to produce the cytokine LIF (Smith et al., 1988), whereas serum was shown to contain BMP4 (Ying et al., 2003); together, these factors were demonstrated to support pluripotency. Based on these and on other studies, ESCs are now routinely cultured in feeder-free conditions known as serum LIF (SL) (Martin Gonzalez et al., 2016; Morgani et al., 2017). ESCs are also commonly grown in a serum-free system that exploits the activity of two small molecule kinase inhibitors – a GSK3 inhibitor and an inhibitor of the ERK regulating kinase MEK – so-called 2i culture (Ying et al., 2008). As this culture system contains a block to FGF/ERK signaling (the MEK inhibitor), PrE differentiation is blocked (Nichols et al., 2009; Hamilton and Brickman, 2014). Although these inhibitors can support a degree of self-renewal in ESCs, LIF was found to be important for supporting undifferentiated ESC growth, leading to the widely used '2iLIF' (2iL) culture medium. ESCs maintained in 2iL acquire a 'naïve' identity that features a more homogeneous Epi-like cell type and is therefore referred to as the 'ground state' of pluripotency (Nichols and Smith, 2009).

Although numerous ESC culture conditions have now been reported, SL culture is still widely used. As an alternative to batch-dependent serum-containing media, activin can be used in conjunction with the GSK3 inhibitor in NACL (N2B27 base media, activin, CHIR and LIF) to produce a defined culture model in which cells exhibit gene expression profiles similar to SL cultured cells (Anderson et al., 2017). In the following sections, we focus on culture models in which the expression profiles have been linked directly to blastocyst development and blastocyst lineage competence. In the discussion that follows, it will become apparent that these different media conditions influence the developmental states captured in culture and that the expansion of these states provides important tools for the dissection of developmental regulatory mechanisms.

ESC heterogeneity and potency

Although naïve ESCs closely resemble cells of the pre-implantation embryo, they are not considered to be totipotent. However, these cells have been observed to contribute to extra-embryonic lineages, albeit at a low frequency (Beddington and Robertson, 1989; Lallemand and Brûlet, 1990; Suemori et al., 1990; Canham et al., 2010; Macfarlan et al., 2012; Morgani et al., 2013; Nigro et al., 2017). Cases of ESCs that are able to contribute both to embryonic and extra-embryonic tissues are summarized in Table S1.

When cultured in conventional SL, ESCs cultures are dynamic and cell states are metastable. In these culture conditions, cells shift between Epi-to-PrE-like states, mimicking the salt-and-pepper expression pattern of the E3.5 ICM (Fig. 1). Unlike ICM cells of the blastocyst, however, these cells are trapped in a dynamic and self-renewing equilibrium between intermediate states in a lineage specification paradigm. Thus, SL cultures contain cells expressing high levels of NANOG, OCT4 and SOX2, which are primed towards Epi specification, and a similar population of cells that continue to express certain pluripotency markers (e.g. OCT4), in addition to low levels of PrE markers (e.g. *Hhex*), that are primed for PrE differentiation. That this dynamic heterogeneity represents functional lineage priming was demonstrated by isolating cells based on the simultaneous expression of a highly sensitive reporter for the early PrE marker *Hhex* [Hhex-Venus (HV)] and ESC cell surface markers (e.g. SSEA1 and PECAM1) and challenging them to differentiate *in vivo* or *in vitro*. In blastocyst and morula injection experiments, ESCs sorted based on simultaneous expression of HV and SSEA1 exhibit distinct patterns of colonization: HV-low- and

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SSEA1-high-expressing cells contribute only to Epi, whereas cells that express high levels of both HV and SEEA1 contribute to the extra-embryonic endoderm, but inefficiently to the Epi (Table S1). When either of these sorted populations are placed back into culture, the original heterogenous population distribution is re-established within 24-48 h (Canham et al., 2010; Illingworth et al., 2016).

When ESCs are grown in chemically defined 2iL conditions they form dome-shaped colonies that homogeneously express naïve markers such as NANOG, OCT4, SOX2, TFCP2L1, TBX3, ESRRB, KLF4 and KLF2 (Ying et al., 2008; Silva et al., 2009; Hall et al., 2009; Martello et al., 2013; Morgani et al., 2013; Martin Gonzalez et al., 2016). However, these cells are not homogeneous with respect to PrE gene expression, and a unique sub-population of these cultures co-expressing Epi and extra-embryonic transcripts can be identified using a combination of the HV reporter and PECAM or SSEA1. Much like early ICM cells, these cells exhibit the remarkable ability to generate all three pre-implantation lineages from a single cell (Table S1), thus demonstrating experimental totipotency (Morgani et al., 2013). Interestingly, under certain 2iL-related human naïve conditions, hESCs also demonstrate remarkable homogeneity of core pluripotency TFs, with a robust population of cells co-expressing PrE determinants, including GATA6 (Chan et al., 2013; Guo et al., 2016, 2017; Linneberg-Agerholm et al., 2019), alluding to an earlier ICM-like state being trapped in human naïve ESCs.

Although the capacity of 2iL-grown ESCs to generate extra-embryonic cell types in chimeras is limited, the chimera contribution observed when TSCs and XEN cells are injected into blastocysts is also not exhaustive (compare data in Table S1 with Kunath et al., 2005 and Tanaka et al., 1998). Moreover, pluripotent cells have long been known to generate PrE (Martin and Evans, 1975; Beddington and Robertson, 1989; D'Amour et al., 2005; Niakan et al., 2013), and it was recently shown that naïve and primed ESCs respond to the same signals to make endoderm, but that naïve ESCs uniquely generate PrE in response to these signals (Anderson et al., 2017; Linneberg-Agerholm et al., 2019). Although the induction of TE from naïve mouse ESCs is normally achieved through induced overexpression of TE-specific factors (Niwa et al., 2005; Blij et al., 2015), when the experimentally totipotent fraction of 2iL-cultured cells (HV-positive cells) is transferred to TSC conditions (Tanaka et al., 1998), trophoblast-like cells appear after several days of differentiation. In fact, single 2iL-cultured HV-positive ESCs can uniquely give rise to all three lineages – Epi, PrE and TE – in clonal *in vitro* differentiation (Morgani et al., 2013). Although the induction of TE from conventional hESCs has been reported as a response to BMP signaling (Amita et al., 2013; Xu et al., 2002), the identity of the resultant cultures is somewhat controversial and includes extra-embryonic mesoderm or amnion (Bernardo et al., 2011; Guo et al., 2020 preprint). However, naïve hESCs are able to differentiate to TE, via an earlier intermediate cell type (Dong et al., 2020; Guo et al., 2020 preprint), and are uniquely competent to produce TSCs similar to those derived from human pre-implantation embryos (Dong et al., 2020). Taken together, these observations suggest that a population within naïve 2iL-grown ESC cultures is experimentally totipotent as it has the capacity to differentiate and contribute to both embryonic and extra-embryonic tissues. Moreover, it has been shown that LIF and the downstream JAK/STAT pathway support both ICM and PrE identity *in vivo* (Do et al., 2013; Morgani and Brickman, 2015). When LIF is added to 2i medium, it induces extra-embryonic gene expression *in vitro* and enhances the potency of ESCs (Morgani et al., 2013; Morgani and Brickman, 2015). This is consistent with the *in vivo* expression pattern of the downstream LIF target KLF4 (Niwa et al., 2009), which is already expressed at the 2C stage and maintained in the ICM, Epi and nascent PrE (Morgani and Brickman, 2015).

In summary, while naïve ESCs are considered pluripotent, the unique capacity of specific fractions of these cultures to either generate extra-embryonic endoderm, or exhibit experimental totipotency as we define it here, suggests that there is continuum in differentiation competence that begins with experimental totipotency and then becomes progressively more restricted. Thus, although traditional ESC cultures exhibit a degree of experimental totipotency, this is likely based on the presence of unique sub-populations in specific culture conditions that resemble intermediates in early embryogenesis. This idea of a continuum of cell states, which includes states that are 'more' than pluripotent, has also been highlighted in another subpopulation of ESC cultures that shares some features with the 2C state, namely 2C-like cells (2CLCs), which we discuss below (and which are discussed at length in the accompanying Primer by Genet and Torres-Padilla, 2020).

2C stage embryos, 2C-specific genes and 2C-like cells

In mice, the first 48 h of post-fertilization development and the concurrent event of zygotic genome activation (ZGA), which initiates at the 2C stage (Fig. 1), is an epigenetic rollercoaster during which the transcriptionally inactive, condensed and hypermethylated chromatin of both the male and female gametes is rapidly unwound, demethylated and repackaged (reviewed by Vastenhouw et al., 2019). Zygotic DNA is relatively hypomethylated (Lee et al., 2014) and histones are depleted of most post-translational modifications (PTMs), resulting in a loose chromatin state (Eckersley-Maslin et al., 2018). In particular, DNA methylation at 5 methyl cytosine (5mC) safeguards the expression of unwanted transcripts (Wiekowski et al., 1993). The removal of 5mC, during ZGA, allows the expression of pseudogenes and repetitive sequences, giving the 2C stage a unique transcriptional signature (Fig. 1). This signature is characterized by the expression of retrotransposons such as endogenous retroviruses (ERVs), long interspaced nuclear elements (LINE-1s) and short interspaced nuclear elements (SINEs) (Kigami et al., 2003; Peaston et al., 2004; Fadloun et al., 2013; Percharde et al., 2018). In addition to these elements, a number of distinct factors are expressed at the 2C stage, including ZSCAN4, TCSTV1/3, EIF1α, TDPOZ2/4 and TMEM92, among others (Falco et al., 2007; Hung et al., 2013; Cerulo et al., 2014; Akiyama et al., 2015; Zhang et al., 2016).

Although such 2C-specific genes are expressed transiently during embryonic development, they have also been observed dynamically in a subpopulation of ESCs *in vitro*. Similar to their *in vivo* counterparts, these rare 2CLCs express high levels of 2C-specific genes, reduced levels of pluripotency markers at the protein level, possibly owing to 2C state-specific translational inhibition (Eckersley-Maslin et al., 2016) and are depleted of chromocenters (Ishiuchi et al., 2015). That these cells bear resemblance to the *in vivo* 2C state is also supported by their distinct metabolic signature, which, like that of the 2C stage, relies more significantly on OXPHOS (Rodriguez-Terrones et al., 2020) and on their capacity to contribute to TE in addition to the ICM. For example, when MERVL-positive 2CLCs cells are injected into morulae, these cells contribute to the placenta, including giant cells, the yolk sac endoderm and the three germ layers of the embryo (Table S1). 2CLCs therefore have been dubbed totipotent (Macfarlan et al., 2012) or, as we define it here, experimentally totipotent.

Given the expanded developmental potential of 2CLCs, a number of recent studies have focused on characterizing these cells and elucidating the role of 2C-specific genes and the factors that regulate

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their expression (reviewed in the accompanying Primer by Genet and Torres-Padilla, 2020). These studies have shown that a number of 2C-specific TFs, including ZSCAN4, are essential regulators of telomere elongation, acting via telomeric recombination or sister chromatid exchange (T-SCE), rather than by promoting an increase in telomerase activity. In ESCs, *Zscan4* knockdown results in culture collapse, followed by a progressive loss of the ESC phenotype and proliferation (Zalzman et al., 2010). This is a result of defective telomere elongation, suggesting that the immortal nature and self-renewing capacity of ESCs would not be possible without a 2C-like state. Consistent with this notion, transient induction of ZSCAN4 expression rescues the reduced developmental potency of high passage ESCs in tetraploid chimeras (Amano et al., 2013) (Table S1).

It has also been shown that the manipulation of various chromatin modifiers can result in increased 2C-specific gene expression in ESCs or the generation of induced 2CLCs (i2CLCs; see also the accompanying Primer by Genet and Torres-Padilla, 2020). Factors controlling 2C-specific gene expression in ESCs include the histone chaperone CAF1, the multifunctional protein KAP1 (also known as TRIM28), the lysine demethylase LSD1 (also known as KDM1A), the lysine methyltransferase G9A, the telomere-associated protein RIF1, the helicase CHD5, the heterochromatin-binding protein HP1, the PRC1 complex component RYBP, the non-canonical PRC1 complex PRC1.6, the EP400-TIP60 complex and the SUMO E3 ligase PIAS4 (Macfarlan et al., 2012; Rowe et al., 2013; Maksakova et al., 2013; Dan et al., 2014; Ishiuchi et al., 2015; Hayashi et al., 2016; Eckersley-Maslin et al., 2016; Rodriguez-Terrones et al., 2018; Yan et al., 2019).

The expression of 2C-specific factors can also be driven by manipulating the potential 2C gene regulatory network (see accompanying Primer by Genet and Torres-Padilla, 2020). For example, the TE-associated TF GATA2 can induce MERVL expression, and the indirect induction of GATA2 via the microRNA *miR-34a* can promote experimental totipotency (Choi et al., 2017) (Table S1). The DUX-family of TFs, which are unique potential regulators of ZGA in eutherian mammals, can also regulate 2C-specific gene expression in ESCs (Hendrickson et al., 2017; Iaco et al., 2017). DUX proteins are homeodomain-containing TFs that are transcribed from genes located in tandem in telomeric or sub-telomeric regions and are some of the earliest transcripts to be induced zygotically. DUX proteins regulate the expression of *Zscan4*, as well as that of other 2C-specific transcripts, and appear to be required for the initiation and maintenance of 2CLCs. *Dux* depletion by siRNA results in impaired 2C transitions, $Dux^{-/-}$ ESCs do not cycle through the 2C state and *Dux* overexpression converts ESC to i2CLCs (Hendrickson et al., 2017; Iaco et al., 2017). Moreover, the regulation of *Dux* via Dppa TFs appears important in promoting this state *in vivo*, and overexpression of DPPA2 and DPPA4 in ESCs also stimulates a 2C-like state (De Iaco et al., 2019). Knockdown of chromatin factors known to repress 2C-specific gene expression, such as CAF1 and KAP1, also results in increased *Dux* expression (De Iaco et al., 2019).

Levels of the maternal factor NELFA have also been associated with Dux expression and, indirectly, the induction of experimental totipotency. NELFA was found to act in combination with TOP2A to regulate *Dux* expression, consistent with the capacity of ESCs expressing high levels of NELFA to contribute both to the ICM and TE in morula injection (Table S1) (Hu et al., 2020). In addition to regulating 2C gene transcription directly, DUX was found to regulate a microRNA cluster (*miR-344*) that contributes to experimental totipotency. Specifically, DUX directly binds to the *miR-344* promoter and activates its expression; *miR-344* then silences ZMYM2 and its partner LSD1, subsequently de-repressing the MERVL LTR, which allows for the transcription of nearby 2C-specific genes. Ectopic expression of either *miR-344* or MERVL via a CRISPR-induction strategy enables ESCs to contribute to both embryonic and extra-embryonic lineages upon morula injection (Yang et al., 2020) (Table S1). Surprisingly, although knockdown of Dux by CRISPR/Cas9 injection into embryos results in a decrease in the expression of some 2C genes and impairs or delays progression in preimplantation development, maternal zygotic *Dux* mutant embryos are viable, although born at slightly below Mendelian ratios, indicating that there is not an absolute requirement for Dux-mediated induction of a 2C state *in vivo* (Iaco et al., 2017; Chen and Zhang, 2019). The requirement for Dux *in vivo* is therefore not as pivotal as its role *in vitro*, suggesting that direct comparisons between the 2C state *in vivo* and *in vitro* 2CLCs in ESC culture should be made cautiously.

Inducing putative experimental totipotency

Following on from the discovery that different experimentally totipotent populations can arise in naïve ESC cultures, several recent studies have focused on identifying culture conditions that enable the expansion of these populations (Table S1 and Fig. 2).

Knockout serum replacement (KOSR) media (Price et al., 1998) promotes expansion of 2CLCs, as indicated by increased levels of ZSCAN4 (Amano et al., 2013; Martin Gonzalez et al., 2016). This media also results in an upregulation of PrE gene expression, resulting in a transcriptome that correlates with early ICM stages. Consistent with the characteristics of putative ICM-like cells, this subpopulation of cells co-expresses antagonistic lineage factors, such as GATA6 (PrE), and NANOG (Epi) (Martin Gonzalez et al., 2016), as well as a combination of the lineage-specific cell-surface markers PDGFRA (PrE) and PECAM (Epi) (Nigro et al., 2017). Moreover, this ICM-like subpopulation of KOSR-cultured cells contributes to both embryonic and extra-embryonic cell types in chimera assays (Martin Gonzalez et al., 2016; Nigro et al., 2017) (Table S1).

A small-molecule screen using a luciferase assay with the ICM-specific distal Oct4 enhancer (Yeom et al., 1996) produced a completely defined culture media for the expansion of experimentally totipotent cells. This minimum cocktail of small molecules includes LIF, CHIR, (S)-(+)-dimethindene maleate (DiM) (a G-protein-coupled receptor inhibitor that appears to illicit its effect on MAPK signaling) and minocycline hydrochloride (MiH) (a PARP1 inhibitor); based on its components, this media is referred to as LCDM. LCDM supports the expansion of colonies with a characteristic dome-shaped morphology in both mouse and human cells lines, and can be used to derive cell lines directly from a blastocyst. Individual LCDM-cultured cells also exhibit extra-embryonic potential: 32% of chimeric embryos injected at the eight-cell stage show both ICM and TE contribution. These cells are therefore referred to as extended pluripotent stem (EPS) cells (Yang et al., 2017a) (Table S1).

Another approach to generate cells with expanded potential is based on inhibiting key pathways involved in lineage specification (e.g. the MAPK, Src and Wnt/Hippo/TNKS1/2 pathways). The use of small molecules for parallel inhibition of these pathways allows cell line derivation from individual blastomeres at different pre-implantation stages ranging from four- to 32-cell embryos. These cell lines, named as expanded potential stem cells (EPSCs), also exhibit properties of experimental totipotency at the single cell level (Yang et al., 2017b) (Table S1). However, transcriptionally, EPSCs are distinct from 2iL ground-state ESCs as they appear to exhibit higher levels of ICM gene expression and share an apparent overall

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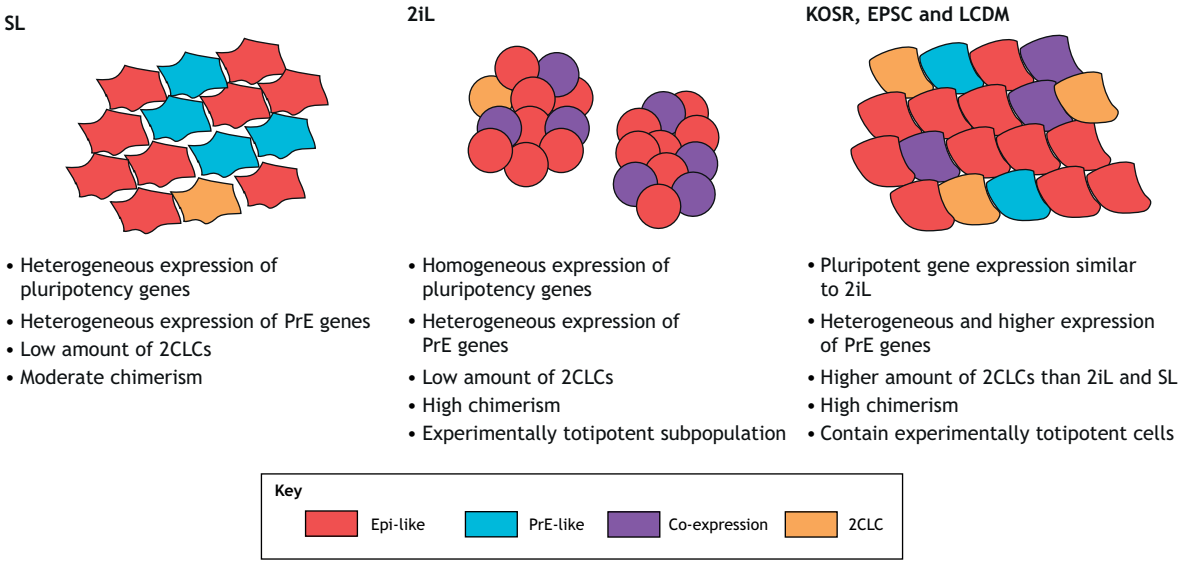

Fig. 2. Schematic illustration of heterogeneity in embryo-derived stem cell cultures. The specific sub-populations that have been identified in different cultures: serum LIF (SL), 2iLIF (2iL), knockout serum replacement (KOSR) and, potentially, in LCDM and expanded potential stem cell (EPSC) media. The characteristics of cells grown under these conditions are also summarized, highlighting that a number of possible cell states could be present in each case and suggesting that varying levels of heterogeneity could explain different observations in different laboratories. 2CLCs, 2C-like cells; Epi, epiblast; PrE, primitive endoderm.

similarity to the four- and eight-cell stages of development (Yang et al., 2017b).

More recent single cell transcriptomic analyses suggest that the transcriptional state of EPSCs might correlate better with that of the E4.5 epiblast, while the state of LCDM-cultured cells resembles that of the E5.5 epiblast (Posfai et al., 2020 preprint). These observations prompted a direct comparison of the potency of these cells in morula aggregations (Posfai et al., 2020 preprint). This study reported that, although these cells can contribute to both Epi and TE at E4.5 (in around 20% of the embryos), they only differentiate properly in the Epi, and cells that localize to the TE do not express the TE marker CDX2. Contribution to extra-embryonic tissues was also analyzed at E6.5 and E12.5, revealing that EPSCs and LCDM-cultured cells contribute to the trophectoderm region at E6.5 but fail to express the trophoblast markers ELF5 and TFAP2C, and continue to express the pluripotency marker OCT4. Moreover, no contribution to the placenta at E12.5 was detected in chimeric embryos from EPSC donors (Posfai et al., 2020 preprint). Thus, although there are examples of experimental totipotency in which lineage differentiation has been demonstrated, this is not always the case; many studies simply used location as a proxy for differentiation when determining the potency of a particular cell population. This study reminds us that cell types are determined not only by their position in the embryo but also by active and appropriate differentiation. Moving forward, it will be important to document experimental totipotency based on both position and cell type identity.

Perhaps these experimental discrepancies are also an indication that experimentally totipotent cells represent a relatively rare population, even in these modified culture conditions (Fig. 2). Thus, as in the case of KOSR and 2iL, these cultures could contain subpopulations of cells that exhibit experimental totipotency but the level of this population may vary depending on slightly different culture conditions in different laboratories. But why is it so difficult to expand these populations? Perhaps these states are inherently unstable or the culture conditions need further optimization. All of the culture conditions described above are expected to have some metabolic impact, but maybe the heightened metabolic requirements of experimental totipotency are difficult to sustain through cell division. However, it is worth noting that naïve pluripotency *in vivo* lasts less than 24 h, whereas experimental totipotency appears to exist stably across five consecutive cell cycles. Given this stability or persistence *in vivo*, it is also possible that the heterogeneity observed in cell culture reflects an as yet undiscovered paracrine interaction that is required to support the consistent expansion of an experimentally totipotent cell type.

Perspectives

In recent years, a number of conditions have been established to support *in vitro* populations that harbor the capacity for both embryonic and extra-embryonic differentiation, or experimental totipotency as we call it. However, is it possible to generate an embryo from these cultures? Will they ever be totipotent in the canonical sense? For many years it has been clear that pluripotent cells have the remarkable capacity for self-organization, as evidenced by embryoid body formation (Martin and Evans, 1975; Brickman and Serup, 2017) or by the formation of more precisely defined structures known as gastruloids (van den Brink et al., 2014). However, it has also been shown that, when naïve ESCs are combined with TSCs and/or XEN cells, these self-organizing aggregates further recapitulate early embryonic development, exhibiting characteristics of the onset of gastrulation, referred to as ETS embryos (Harrison et al., 2017) or ETX embryoids (Zhang et al., 2019). Aggregations based on defined cell numbers and microwell technology have also been shown to give rise to blastocyst-like structures or blastoids (Rivron et al., 2018; Sozen et al., 2019). As the capacity of a cell to generate a complete intact embryo would be defined as canonical totipotency, the capacity of single cell type to generate blastocyst-like structures such as EPS blastoids (Li et al., 2019) and blastocyst-like cysts (iBLCs) (Kime et al., 2019) edges toward this unambiguous definition. Blastoids, ETX embryoids and iBLCs have all been shown to be capable of recapitulating several features of implantation, although post-implantation development is not supported (Kime et al., 2019; Li

et al., 2019; Rivron et al., 2018; Sozen et al., 2019; Zhang et al., 2019) (Table S1). Although these recent attempts to recapitulate embryo development have not achieved canonical totipotency or the production of a genuine embryo, the increasing efforts to expand experimental totipotent cells and their application to the generation of synthetic embryos suggests that the grey area between canonical and experimental totipotency may soon disappear.

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Table S1. Summary of published work on cell potency for *in vitro* cell lines

Cells used	Media	*In vitro* differentiation assays	Method of chimera generation	Numbers of cells introduced into host embryos	Observed contribution to multiple lineages at the blastocyst stage	Observed contribution to later embryonic/fetal and extra-embryonic lineages	Chimeric offspring	Reference(s)
ESCs (CP1 and CP3)	Serum (on feeders)	N.A.	Blastocyst injection	10-15 cells	N.A.	At E10.5, 15/16 chimeras showed ESC contribution to embryonic tissues, and three of these also exhibited VE or PE contribution. An additional two of these showed trophoblast contribution, and one embryo showed contribution to all three lineages. One embryo showed trophoblast contribution only.	N.A.	Beddington and Robertson (1989)
				Single cells	N.A.	At E10.5, 12/12 chimeras show ESC contribution to the embryonic tissues, one of them also showed contribution to the PE.	N.A.	
ESCs (N1, D3 ESCs; LacZ promoter trap)	Serum (on feeders)	Embryoid bodies	Blastocyst and morula injection	15-20 cells	34/53 blastocysts showed ESC contribution to the ICM and TE. 13/53 showed contribution to the ICM only and 6/53 to the TE only.	From E5.5 to E9.5, ESC contribution to the embryonic, but not extra-embryonic, lineages was observed.	N.A.	Lallemand and Brûllet (1990)
				Single cell	6/14 blastocysts show ESC contribution to the ICM, 8/14 show contribution to TE only and 0/14 show contribution in both lineages.	N.A.	N.A.	
ESCs (Hhex-Venus; CAG-βgeo), SSEA1$^+$	SL	Embryoid bodies	Morula aggregation and blastocyst injection	N.A.	N.A.	At E6.5, 12/120 embryos derived from Hhex$^+$/SSEA1$^+$ ESCs showed VE/PE contribution with modest contribution to the embryo proper and 65/120 showed low-moderate embryonic contribution, whereas 52/69 Hhex$^-$/SSEA1$^+$ ESCs showed high levels of embryonic contribution.	N.A.	Canham et al. (2010)
ESCs (MERVL-Tomato; CMV-GFP or EF1α-GFP)	SL, KOSR, 2iL	N.A.	Morula and blastocyst injection	4 cells	From morula injections, 3/5 chimeric blastocysts showed MERVL$^+$ ESC contribution to both the ICM and TE. 1/5 showed contribution to the TE only.	From blastocyst injections at E12.5, MERVL$^+$ ESCs contributed to the embryonic lineages, yolk sac and placenta.	N.A.	Macfarlan et al. (2012)
ESCs (Zscan4-Emerald)	SL	N.A.	Blastocyst injection	10-15 cells and single cells	N.A.	N.A.	Zscan4$^-$ ESCs support live-born chimeric mice at high frequencies (75% for multiple cells, 31% for single cells), whereas Zscan4$^+$ ESCs support reduced levels of chimera formation (31% for multiple cells, 0% for single cells). Multiple ESC lines were tested with significant *n* values.	Amano et al. (2013)
ESCs (Zscan4-ERT2 overexpression)	SL	N.A.	4N blastocyst injection	10-15 cells and single cells	N.A.	N.A.	Overexpression of Zscan4-ERT2 restored the ability to form chimeras in high passage ESCs in multiple clones, with up to 43% live-born chimeric mice, compared to 0-3% for controls. For single-cell injections, a similar enhancement was observed (5% vs 0-1% for controls). Multiple ESC lines were tested with significant *n* values.	
ESCs (Hhex-Venus;CAG-LacZ), PECAM1$^+$ and (Hhex-Venus;H2B-Tomato), PECAM1$^+$	2iL	Trophoblast and endoderm differentiation	Morula aggregation and morula injection	8-10 cells	3/7 blastocysts generated from HhexVenus$^+$ ESCs show contribution to embryonic and extra-embryonic lineages.	At E6.5, 30/55 chimeric embryos generated with HhexVenus$^+$ 2iL ESCs show contribution to both embryonic and extra-embryonic lineages. Chimeric embryos from HhexVenus$^-$ 2iL ESCs also showed embryonic and extra-embryonic contribution, but at a much lower rate (18/60 embryos). At E9.5, 3/8 embryos derived from HhexVenus$^+$ ESCs showed placenta and visceral yolk sac contribution (assessed by histology).	N.A.	Morgani et al. (2013)
				Single cells	N.A.	At E6.5, 13/23 chimeric embryos from single HhexVenus$^+$ ESCs showed contribution to both the Epi and VE or Epi and TE. Three of them showed contribution to all three lineages. Staining for KRT7 (TE) and GATA6 (VE and PE).	N.A.	
ESCs (H2B-Tomato)	SL and 2iL	N.A.	Morula injection	3-8 cells	30/30 blastocysts showed ESC contribution to Epi, 2/30 to the TE and 3/30 to the PrE (ESCs in the TE and PrE did not co-stain with CDX2 and SOX17, respectively).	N.A.	Two chimeric embryos out of six pups from SL-injected embryos. Live-born chimeras were generated following imaging to show that embryos survived time -lapse.	Alexandrova et al. (2016)

Cells used	Media	*In vitro* differentiation assays	Method of chimera generation	Numbers of cells introduced into host embryos	Observed contribution to multiple lineages at the blastocyst stage	Observed contribution to later embryonic/fetal and extra-embryonic lineages	Chimeric offspring	Reference(s)
ESCs (CAG-H2B-Tomato and CAG-Kozac-Venus) derived from F1 129S6;C57BL/6N	2iL and KOSR	Trophoblast and endoderm differentiation	2C embryo injection	Single cells	N.A.	N.A.	Single ESCs grown in 2iL support high contribution chimera formation (up to 100%, 26/87). ESCs grown in KOSR also generate high contribution chimeras (3/8). ESC-derived chimeric mice were judged by coat color, organ composition and germ-line competence.	Martin Gonzalez et al. (2016)
			Morula injection	Single cells (also 5 cells, not included in this table).	N.A.	N.A.	Single ESCs grown in 2iL support high contribution chimera formation (up to 100%, 4/9). ESCs grown in KOSR also generate high contribution chimeras (2/15). ESC-derived chimeric mice were judged by coat color, organ composition and germ-line competence.	
ESCs (EF1α-GFP), PECAM1^{+}, PDGFRA^{+} and double positives)	KOSR	Trophoblast and endoderm differentiation	Blastocyst injection	6-8 cells	N.A.	At E6.5, 4/8 chimeric embryos generated with double-positive cells showed contribution to both Epi and PrE derivatives. 15/18 chimeric embryos generated from PDGFRA^{+}/PECAM1^{-} ESCs showed contribution to PrE derivatives, and 12/12 chimeric embryos from PDGFRA^{-}/PECAM1^{+} ESCs showed contribution to Epi.	N.A.	Nigro et al. (2017)
ESCs (miR34a$^{-/-}$; GFP)	SL and 2iL	Embryoid bodies	Morula and blastocyst injection	4 cells	28/46 blastocysts from miR34a$^{-/-}$ ESCs showed contribution to the ICM and TE, 2/46 showed only TE contribution. Blastocysts from wild-type ESCs only contributed to the ICM (23/23).	N.A.	N.A.	Choi et al. (2017)
				Single cells	21/61 blastocysts from miR34a$^{-/-}$ ESCs showed contribution to the ICM and TE, and 20/61 showed contribution only to the TE.	N.A.	N.A.	
				10-15 cells	N.A.	Chimeras generated with miR34a$^{-/-}$ ESCs had both embryonic and extra-embryonic contribution: 4/10 embryos at E9.5, 5/8 embryos at E12.5 and 12/15 at E14.5. Wild-type ESCs did not contribute to extra-embryonic tissues at any stage. TPBPA and MTP1 staining used to identify trophoblast derivatives.	N.A.	
mEPS cells (LCDM-EPS cells; CAG-tdTomato) and hEPS cells (LCDM-EPS cells; CAG-tdTomato)	LCDM	N.A.	8C embryo injection	Single cells	86/261 blastocysts showed EPS cell contribution to the ICM and TE. Staining for NANOG and OCT4 (ICM), GATA3 and CDX2 (TE). Single mESCs only contributed to the ICM.	Chimeras generated with single EPS cells showed both embryonic and extra-embryonic contribution: 21/90 embryos at E10.5, 10/63 embryos at E12.5 (with one embryo with solely extra-embryonic) and 13/94 embryos at E17.5. CK8, PLF and TPBPA staining used to identify trophoblast derivatives.	59/113 live-born chimeras with high levels of germ-line transmission (>67%).	Yang et al. (2017a)
			Morula and blastocyst injection	10-15 TSC-like and ESC-like EPS cells (derived from embryos injected with a single EPS cell at the 8-cell stage)	N.A.	EPS-ESCs contributed only to the embryos; EPS-TSCs contributed only to placental tissue (at E13.5).	N.A.	
			4N blastocyst injection	Single mEPS cells	N.A.	N.A.	Seven mEPS single cell-derived mice were live-born (out of 311 injected embryos).	
			8C embryo injection	Single hEPS cells	51/345 blastocysts showed hEPS cell contribution to mouse ICM and TE, 24/345 showed hEPS contribution only to the TE, and 43/345 showed only ICM contribution. Human ESCs or primed iPSCs did not contribute to mouse blastocysts (n =143). Staining for NANOG and OCT4 (ICM), GATA3 and CDX2 (TE).	N.A.	N.A.	

Cells used	Media	*In vitro* differentiation assays	Method of chimera generation	Numbers of cells introduced into host embryos	Observed contribution to multiple lineages at the blastocyst stage	Observed contribution to later embryonic/fetal and extra-embryonic lineages	Chimeric offspring	Reference(s)
			Morula and blastocyst injection	10-15 hEPS cells	N.A.	Chimeras generated with hEPS cells at E10.5 showed contribution to the mouse embryo (24/54) or placenta (9/54), or both (6/54).	N.A.	Yang et al. (2017a)
EPSCs (CAG–mCherry, EF-1α-H2B–mCherry or CAG-H2B–mCherry)	EPSC	TSC and XEN cells derived from EPSCs. Additional *in vitro* differentiation to embryonic lineages.	Morula and 8C embryo injection	6-8 cells	9/17 blastocysts had EPSC contribution to both the ICM and TE. 1/17 showed TE contribution only. ESCs showed only ICM contribution and CDX2 staining was used to identify TE.	At E6.5, 78/225 embryos generated with EPSCs showed contribution to both the embryo proper and the extra-embryonic ectoderm. 4/225 showed only extra-embryonic contribution. ELF5 staining was used to identify extra-embryonic ectoderm. At E14.5, chimeras generated from EPSCs showed contribution to the yolk sac and placenta, based on histology (placental staining for TFAP2C). Donor cells were also sorted from chimeric placentas, followed by RT-PCR and staining for specific markers (TFAP2C, GCM1, Ezrin and CK7).	Germ-line contribution from one male chimera.	Yang et al. (2017b)
			8C embryo injection	Single cell	N.A.	At E14.5, 28/190 chimeras were produced from single EPSCs. Some showed contribution to the extra-embryonic lineages.	N.A.	
Blastoids (generated from a combination of ESCs and TSCs)	SL+TX media in microwells	Derivation of ESCs (2iL) and TSCs from blastoids	Blastoid transfer	25 blastoids	N.A.	10% of blastoids generated deciduas (*n* =5 mice). Staining of deciduas and inside structures for CDX2, ELF5, TEAD4, ASCL2, HAND1, PLF and ALDH3A1 at E7.5.	N.A.	Rivron et al. (2018)
			Morula injection of blastoid-derived TSCs and ESCs	12-15 cells	N.A.	At E6.5, chimeras were generated with blastoid-derived ESCs contributing to the Epi and blastoid-derived TSCs contributing to the TE. Placental contribution of blastoid-derived TSCs is also shown at E11.5.	N.A.	
EPS-blastoids (generated from LCDM mEPS cells)	EPS-blastoid media (50%KSOM, 25% N2B27 and 25% TSC) in aggrewells	Derivation of ESCs (2iL), TSCs and XEN cells from EPS-blastoids	EPS-blastoid transfer	20 blastoids	N.A.	7.3% of EPS-blastoids generated deciduas (*n* =300). Analysis of post-implantation stages indicated that 4/9 deciduas contained malformed embryo-like structures. Stained for CK8, GATA4, EOMES and OCT4.	N.A.	Li et al. (2019)
			Blastocyst injection of ESCs, TSCs and XEN cells derived from EPS-blastoids	15 cells	N.A.	In post-implantation embryos, EPS-blastoid-derived TSCs generated chimeric placental tissues (CK8 staining), while EPS-blastoid-derived XEN cells contributed to the yolk sac.	EPS-blastoid-derived ESCs could generate adult chimeric mice, although with low levels of chimerism judged by coat color.	
EPS-blastoids (generated from TSCs and LCDM mEPS cells)	LCDM+TX media in aggrewells	N.A.	EPS-blastoid transfer	N.A.	N.A.	Decidualization assessed 4 days post-transfer. Staining of deciduas and inside structures for PTGS2, Ki67, CDX2, KRT18, PLF, SOX2 and EOMES.	N.A.	Sozen et al. (2019)
ETX-embryoids (generated from a combination of ESCs, TSCs and XENs)	ETX-embryo medium (39% RPMI, 39% DMEM, 17.5% FBS)	N.A.	36 h ETX-embryoid transfer to E3.5 dpc pseudo-pregnant females	20 embryoids	N.A.	87/97 mice contained implantation sites after ETX-embryoid-transfer. 20-30% of ETX-embryoids generated deciduas (*n* =5 experiments). 48 hours post transfer, 2/20 deciduas contained embryo-like structures. Staining of deciduas and inside structures for COX2, PL1, CK, GATA4 and LAMININ.	N.A.	Zhang et al. (2019)
iBLC (generated by reprogramming mEpiSCs that contain a MERVL-RFP reporter)	CTSFES medium (DMEM F12, N2B27 and ascorbic acid)	ESC- and TE-like cells	iBLC transfer	N.A.	N.A.	10/149 iBLCs generated deciduas. 24/186 deciduas formed when iBLCs were co-transferred with blastocysts. Histology of deciduas and staining for PL1, TPBPA and TROMA1.	N.A.	Kime et al. (2019)
ESCs [Dppa2 overexpression (OE) and Pias4 knockout (KO); mRuby2]	SL	N.A.	8C embryo injection	4 cells	16/40 blastocysts showed ESC contribution to the TE and ICM, and 3/40 to the TE only from Dppa2 OE cells. 16/42 blastocyts had dual contribution from Pias4 KO cells. No contribution to the TE was reported for wild-type ESCs (*n* =19).	N.A.	N.A.	Yan et al. (2019)
2CLCs (miR-344-GFP or MERVL-GFP both activated by CRISPR$_{SAM}$)	SL	N.A.	8C embryo injection	Single cells (or up to 3)	11/74 blastocysts had ESC contribution to the TE and ICM from ESCs expressing *miR-344,* with 2/74 exhibiting only TE contribution. 10/66 blastocysts showed ESC contribution to the TE and ICM from ESCs in which MERVL was activated. No TE contribution observed from WT ESC (*n* =66).	At E12.5, chimeras from miR-344- and MERVL-activated ESCs contributed to the placenta (7/11 and 8/11, respectively). Staining for TBPA and PLF was used to identify trophoblast derivatives.	N.A.	Yang et al. (2020)

Cells used	Media	*In vitro* differentiation assays	Method of chimera generation	Numbers of cells introduced into host embryos	Observed contribution to multiple lineages at the blastocyst stage	Observed contribution to later embryonic/fetal and extra-embryonic lineages	Chimeric offspring	Reference(s)
ESCs (NELFA-Strep-HA-P2A-EGFP reporter; mCherry), sorting NELFA^{high} and NELFA^{low} ESCs	SL	N.A.	8C embryo or E3.25 blastocyst injection	5-7 cells	12/73 blastocysts showed contribution from NELFA^{high} ESCs to both the ICM and TE. NELFA^{low} cells contributed only to the ICM (n=74). Staining for CDX2 was used to identify the TE.	N.A.	N.A.	Hu et al. (2020)
ESCs (H2B-eGFP)	2iL, EPSC (or L-EPSC), and LCDM (or D-EPSC)	TSC	Morula aggregation	6-8 cells	Approximately 20% of chimeric embryos had both Epi and TE-localized contribution from cells cultured in EPSC media (n=34) and LCDM (n=40). Donor cells did not stain for the TE marker CDX2.	At E6.5, approximately 5% of chimeras derived from cells cultured in 2iL or LCDM media (n=14 and 23, respectively) showed Epi and TE contribution, whereas cells grown in EPSC media had higher levels (20%) of dual lineage contribution (n=26). None of the TE-localized contributing cells expressed either TFAP2C or ELF5 and continued to express OCT4. At E12.5, in chimeras from cells grown in EPSC media, there was no evidence of placental contribution (staining for TFAP2C).	N.A.	Posfai et al. (2020)

This table is not meant to be exhaustive, but includes all reports of ESCs or ESC-like cells with extra-embryonic potential that we are aware of. It includes the experiment type, n values for experiments, stages assessed and types of analysis. Not all experiments in every referenced paper are listed, only those directly relevant to the discussion in this Primer. 2C, 2-cell stage embryo; 2CLCs, 2C-like cells; 2iL, 2iLIF; 4N, tetraploid; Epi, epiblast; EpiSCs, epiblast-stem cells; EPS cells, extended pluripotent stem cells generated in LCDM media; EPSCs, expanded potential stem cells; ESC, embryonic stem cell; hEPS, human EPS; hESCs, human ESCs; iBLC, induced blastocyst-like cysts; ICM, inner cell mass; KOSR, knockout serum replacement; m, mouse; N.A., not assessed; PE, parietal endoderm; PrE, primitive endoderm; SL, serum LIF; TE, trophectoderm; TSC, trophoblast stem cell; TX, TSC media; VE, visceral endoderm; XEN, extra-embryonic endoderm stem cell.