The molecular and cellular features of 2-cell-like cells: a reference guide

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Abstract

Currently, two main cell culture models predominate pluripotent stem cell research: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Thanks to their ability to contribute to and form all tissues within the body, ESCs and iPSCs have proven invaluable in understanding pluripotent states, early embryonic development and cell differentiation, as well as in devising strategies in regenerative medicine. Comparatively, little is known about totipotency - a cellular state with greater developmental potential. In mice, only the zygote and the blastomeres of the 2-cell stage embryo are truly totipotent, as they alone can develop to form the embryo and all its supportive extra-embryonic tissues. However, the discovery of a rare sub-population of cells in murine ESC cultures, possessing features of 2-cell embryo blastomeres and expanded cell fate potential, has provided a biochemically tractable model to enable the *in vitro* study of totipotency. Here, we summarize current known features of these 2-cell-like cells (2CLCs) in an effort to provide a reference for the community, and to clarify what we know about their identity so far.

Introduction

"What am I?" wonders the 2-cell-like cell.

Over the last two decades, extensive research in the pluripotency field has allowed the discovery and characterization of the core components of the pluripotency network. This has enabled, amongst other things, the groundbreaking generation of induced pluripotent stem cells (iPSCs) from somatic cells (Takahashi and Yamanaka, 2006). These and other *in vitro* models of pluripotency, such as embryonic stem cells (ESCs), have proven invaluable for basic research, as well as for more applied biological research and stem cell therapies (Hackett and Surani, 2014; Nichols and Smith, 2009; Ying et al., 2008). By contrast, *in vitro* models of totipotency are lacking and we therefore have a more limited understanding of the totipotent state.

In terms of developmental potential, a totipotent cell differs from a pluripotent cell by its ability to develop into an entire organism by itself (see Box 1 and accompanying piece Riveiro and Brickman 2020). Totipotency is established concomitantly with epigenetic reprogramming after fertilization. The zygote and blastomeres of the 2-cell stage mouse embryo are totipotent; they have the ability to give rise to an embryo and all of its supportive extra-embryonic tissues. In contrast, four-cell stage blastomeres, while highly plastic, are not totipotent as they are unable, when isolated alone, to give rise to a full embryo (Rossant, 1976; Tarkowski and Wróblewska, 1967). Indeed, individual 4-cell and 8-cell blastomeres can lead to the birth of a pup, but only when aggregated with other, supporting cells (Zhang et al., 2018). This therefore indicates that the totipotent state is a transient state, making it difficult to study experimentally. However, the recent discovery of 2-cell-like cells (2CLCs), named after their shared features with the 2-cell embryo, has provided the possibility to establish an *in vitro* cell culture model of totipotency (Macfarlan et al., 2012).

2CLCs were first identified by the Pfaff lab (Macfarlan et al., 2012). The discovery of 2CLCs followed experiments in which ESCs were 'labelled' using promoters of repetitive elements, specifically those expressed during zygotic genome activation (ZGA) in the mouse, which occurs primarily at the late 2-cell stage. Macfarlan and colleagues discovered a rare population of cells expressing retrotransposons from the Murine Endogenous Retrovirus with Leucine tRNA primer (MERVL) family and their corresponding Long Terminal Repeat (LTR) promoter (Mt2_mm), which is in fact the most highly transcribed retrotransposon family at the 2-cell

stage (Macfarlan et al., 2012). Earlier work, had indeed identified MERVL in chimeric transcripts of 2-cell stage embryos, and proposed that MERVL LTRs could function as alternative promoters of host genes during ZGA (Peaston et al., 2004). In addition to MERVL expression, 2CLCs share many transcriptional features with the 2-cell embryo, hence their name. Because ZGA occurs at the late-2-cell stage in the mouse embryo, many ZGA genes are considered to be markers of 2CLCs. Another major feature of 2CLCs is the down-regulation of the pluripotency factor OCT4, as observed by immunostaining (Macfarlan et al., 2012). This contrasts to pluripotent ESCs from which 2CLCs arise, which are characterized by the expression of the pluripotency network, namely OCT4, NANOG and SOX2 (Chambers and Tomlinson, 2009).

Most importantly, 2CLCs are thought to possess an expanded cell fate potential, which must not be confused with Extended Potential Stem (EPS) cells (see Box 2). They have been suggested to contribute to both embryonic and extraembryonic lineages, including trophectoderm (TE) in chimaera assays, and display increased reprogramming efficiency upon nuclear transfer (Choi et al., 2017; Ishiuchi et al., 2015; Macfarlan et al., 2012). According to the broad definition of totipotency, 2CLCs have therefore been referred to as being 'totipotent' in several studies. In accordance with the stringent definition of totipotency, however, we refer to 2CLCs as being 'totipotent-like'. Regardless, it is clear that 2CLCs provide a potential cell culture model to study totipotency and constitute a much-needed tool for investigating epigenetic reprogramming, ZGA and the developmental biology of the earliest stages of mammalian embryogenesis.

Here, we aim to provide an up-to-date list of 2CLC characteristics, including their transcriptional, chromatin, and metabolic features. 2CLCs emerge spontaneously in ESCs, albeit at very low frequency (~0.1 to 0.4%). However, recent work has described conditions that increase the frequency of 2CLC reprogramming (Choi et al., 2017; Ishiuchi et al., 2015; Hu et al., 2020; Rodriguez-Terrones et al., 2020). We also discuss these cells in brief but refer to them as "induced 2CLCs" as opposed to endogenously occurring 2CLCs.

Known characteristics of 2CLCs

To date, 2CLCs have been shown to exhibit several characteristic features, which we discuss below and summarize in **Table 1**. We have included a discussion of ESCs in order to highlight the differences and similarities between the two cell types. Furthermore, the 2-cell stage mouse

preimplantation embryo serves here as a ground truth for totipotency, allowing us to compare 2CLCs with truly totipotent cells.

Transcriptional features

2CLCs down-regulate pluripotency factors

OCT4, SOX2 and NANOG are transcription factors of the core pluripotency network (reviewed in Chambers and Tomlinson, 2009). OCT4 is dispensable for pre-implantation development (Wu and Schöler, 2014), but plays a critical role in the maintenance of pluripotency in the embryo (Nichols et al., 1998), while a correct dose of SOX2 is critical during the totipotency to pluripotency transition in embryos (Pan and Schultz, 2011). In line with this, OCT4, NANOG and SOX2 are required for the establishment and maintenance of pluripotency and self-renewal in ESCs (Fong et al., 2008; Pan and Thomson, 2007; Young, 2011; Zhang and Cui, 2014). However, while pluripotent ESCs and cells of the inner cell mass (ICM) of the blastocyst robustly express OCT4, immunostaining analyses indicate that 2-cell stage embryos lack OCT4 protein (Do et al., 2013; Macfarlan et al., 2012). *Sox2* and *Nanog* are also expressed at very low levels in the 2-cell embryo, but their expression levels increase as development proceeds and are highest at the blastocyst stage, where they become restricted to the epiblast (Avilion et al., 2003; Komatsu and Fujimori, 2015; Silva et al., 2009). 2CLCs, in contrast to ESCs but similar to the 2-cell stage embryo, display undetectable levels of OCT4, SOX2 and NANOG proteins (Macfarlan et al., 2012; Rodriguez-Terrones et al., 2018), even though their respective mRNAs are expressed. This could be explained by potential post-translational regulation of these proteins, which remains to be studied.

2CLCs express genes from the embryonic program at the 2-cell stage

2CLCs are called so because a significant part of their transcriptome overlaps with that of 2-cell stage embryos (Eckersley-Maslin et al., 2016; Ishiuchi et al., 2015; Macfarlan et al., 2012; Rodriguez-Terrones et al., 2018). Below and in **Table 1**, we highlight a few gene families that are expressed in 2-cell embryos and that are most commonly cited for the identification of 2CLCs: *Zfp352*, *Eif1a*, *Eif1a-like* cluster and the *Zscan4* cluster (Cerulo et al., 2014; Eckersley-Maslin et al., 2016; Ishiuchi et al., 2015; Macfarlan et al., 2012).

ZFP352 is a Krüppel-like factor that is specific to ZGA in the mouse but its function in early embryogenesis is unknown (Pei and Grishin, 2013). EIF1A (also known as eIF-4C) is a translation initiation factor that is transiently expressed during ZGA in mice and is thought to

promote translation, but its precise function in the 2-cell embryo or 2CLCs is also not known (Davis et al., 1996; Zhang et al., 2006). The *Eif1a-like* cluster comprises 10 genes and 9 pseudogenes expressed primarily in 2-cell stage embryos (Hung et al., 2013), but their in-depth characterization in the context of the 2-cell embryo and 2CLCs remains to be investigated.

The *Zscan4* (zinc finger and SCAN domain containing protein 4) cluster comprises 6 genes (*Zscan4a-f*) encoding paralogous C2H2-type zinc fingers that are specifically expressed during ZGA in mouse late 2-cell embryos (Falco et al., 2007). RNA interference experiments revealed that embryos depleted of ZSCAN4 display reduced rates of blastocyst formation and fail to hatch and expand, indicating a role for ZSCAN4 during early development. In ESCs, ZSCAN4 regulates telomere elongation and genomic stability by preventing telomere shortening in ESCs (Falco et al., 2007; Zalzman et al., 2010). *Zscan4d* is the predominant transcript in the late 2-cell stage embryo while *Zscan4c* is predominant in ZSCAN4-positive cells in culture. Because of the high sequence homology between the paralogs, the literature often refers to them simply as to '*Zscan4'*, regardless of the specific paralog transcript, and current anti-ZSCAN4 antibodies do not distinguish amongst the proteins. *Zscan4* expression at both the mRNA and protein level is another 2CLC feature (Macfarlan et al., 2012; Rodriguez-Terrones et al., 2018). However, without MERVL reactivation, ZSCAN4 alone is not a marker of 2CLCs by itself. Indeed, the ZSCAN4-positive state is transient and more abundant than the 2CLC population: approximately 5% of ESCs are ZSCAN4-positive, while the population of 2CLCs constitutes between 0.1-0.5% of ESCs. ZSCAN4-positive cells have a transcriptional profile intermediate between that of ESCs and 2CLCs (Rodriguez-Terrones et al., 2018). Indeed, PCA analyses and computational modelling of single cell expression datasets revealed an intermediate cluster of cells distinct from 2CLCs, which do not express MERVL but express the *Zscan4* genes. This intermediate *Zscan4*-positive/MERVL-negative cluster retains its intermediate identity between ESCs and 2CLCs even when excluding the expression of the 2CLC MERVL-driven reporter and the expression of *Zscan4*, suggesting that *Zscan4*-positive cells are distinguished from ESCs and 2CLCs by their whole transcriptomic profile and not solely by the expression of *Zscan4* and MERVL. Furthermore, the chromatin accessibility landscape of ZSCAN4-positive cells is distinct from that of 2CLCs (Eckersley-Maslin et al., 2016; Rodriguez-Terrones et al., 2018; Zalzman et al., 2010). 2CLCs emerge preferentially from this ZSCAN4-positive population of cells, suggesting that the main pathway for 2CLC reprogramming uses ZSCAN4 as intermediate cellular state, although it may not be the only path (Rodriguez-Terrones et al., 2018).

2CLCs express and can be induced by the transcription factor DUX

DUX (*Duxf3*) is a transcription factor expressed during the early 2-cell stage, prior to major ZGA. DUX binds to the promoters of many ZGA-genes and is thought to regulate their expression in both 2-cell embryos and 2CLCs (see also accompanying piece Riveiro and Brickman 2020). Accordingly, DUX is necessary for the correct execution of ZGA in mouse embryos (Fu et al., 2019; Iaco et al., 2017; Iaco et al., 2019) and is necessary and sufficient to induce 2CLCs (Hendrickson et al., 2017; Iaco et al., 2017). DUX is also highly expressed in 2CLCs compared to ESCs (Iaco et al., 2017). Interestingly, the human orthologue, DUX4, binds to the promoters of early ZGA genes and HERVL elements - the human counterparts of MERVL elements - in human iPSCs (Iaco et al., 2017).

Of note, it was recently shown that 2CLCs express high levels of the maternal factor, negative elongation factor A (NELF-A), relative to ESCs, and that NELF-A seems to act upstream of DUX during 2CLC reprogramming (Hu et al., 2020). While this study provides insight into the mechanistic role of NELF-A in the generation of 2CLCs, its role during preimplantation development remains to be investigated.

2CLCs express repetitive elements characteristic of 2-cell stage embryos

MERVL transcripts are 300-fold times more abundant in the 2-cell stage embryo than in the oocyte (Macfarlan et al., 2012; Svoboda et al., 2004). Furthermore, around 307 ZGA genes consist of chimeric transcripts with MERVL-LTRs, in which the 5' LTR acts as an alternative promoter for these host genes (Macfarlan et al., 2012; Peaston et al., 2004). Hence, MERVL reactivation at the 2-cell stage is thought to drive the expression of some ZGA genes. It should be noted, however, that not all totipotent cells express high levels of MERVL elements. For example, MERVL transcript levels in the zygote are very low relative to those in the 2-cell stage, and these transcripts are presumably of maternal origin. The developmental window during which MERVL elements are expressed is short: MERVL elements are significantly downregulated from the 4-cell stage, being expressed at a level comparable to that in the zygote. Notably, 2CLCs can be identified by positive immunostaining for the GAG protein, which is encoded by the *gag* gene - a gene that is present in nearly half of all MERVL repeats. 2CLCs are also routinely identified using a '2C' reporter construct, which is essentially a MERVL-LTR driving the expression of a fluorescent reporter.

2CLCs also reactivate transcription of major satellite repeats that constitute pericentromeric chromatin (Ishiuchi et al., 2015), which is a feature of the zygote and 2-cell embryos (Probst et al., 2010; Puschendorf et al., 2008; Santenard et al., 2010). In the embryo, major satellites are necessary for pericentromeric heterochromatin reorganization at the late 2-cell stage (Casanova et al., 2013). In ESCs, few major satellite transcription foci can be observed by RNA-FISH under certain culture conditions, whereas 2CLCs exhibit a dramatic increase in the number of major satellite foci (Ishiuchi et al., 2015; Tosolini et al., 2018). This is in line with the view that 2CLCs decondense their heterochromatin (discussed below).

Chromatin and nuclear organization features

Histone mobility

Histones are rather immobile proteins once incorporated into chromatin. However, Fluorescence Recovery After Photobleaching (FRAP) experiments performed in nuclei of 2-cell embryos has shown that these totipotent cells display unusually high core-histone mobility (in the case of histones H3.1, H3.2 and H2A), which decreases in pluripotent cells at later stages (Bošković et al., 2014; Ooga et al., 2016). 2CLCs also display high core-histone mobility (for histones H3.1 and H2A), suggesting that histone mobility may be linked to a greater cellular plasticity (Bošković et al., 2014; Ishiuchi et al., 2015).

Chromocenters

Chromocenters are comprised of the centromeric regions from several chromosomes, which come together in the 3D nuclear space and typically appear as dense nuclear puncta when visualized upon DNA staining. In mice, chromocenters are established after ZGA, from the late 2-cell stage onwards, and become clearly consolidated by the 8-cell stage (Aguirre-Lavin et al., 2012; Probst et al., 2007). Hence, the zygote and early 2-cell stage embryo do not have defined chromocenters. Likewise, 2CLCs do not have chromocenters, contrary to the situation in ESCs (Ishiuchi et al., 2015). Of note, ZSCAN4-positive cells also have decondensed pericentromeric chromatin (Akiyama et al., 2015). This suggests that chromocenter decondensation might occur before 2CLC reprogramming or during the ZSCAN4-positive intermediate state. However, loss of chromocenters alone cannot induce 2CLCs, and their influence on cellular plasticity, if any, remains to be tested (Ishiuchi et al., 2015).

Chromatin accessibility

Southern blot coupled with MNase I digestion analysis revealed that 2CLCs have increased chromatin accessibility at MERVL elements (Ishiuchi et al., 2015). Genome-wide ATAC-seq profiling indeed demonstrated that this is a distinctive feature of 2CLCs that clearly distinguishes them from ZSCAN4-positive cells (Eckersley-Maslin et al., 2016; Rodriguez-Terrones et al., 2018). The open chromatin landscape of 2CLCs resembles that of the 2-cell stage embryo (Hendrickson et al., 2017; Wu et al., 2016): ATAC-seq peaks that appear in 2CLCs but not ESCs are accessible peaks in 2-cell stage embryos (Hendrickson et al., 2017). Importantly, an unbiased clustering analysis of genome-wide ATAC-seq signals clustered 2CLCs together with 2-cell-stage embryos (Hendrickson et al., 2017). Thus, 2CLCs are characterized by accessible chromatin at MERVL elements and acquire an open/closed chromatin landscape resembling that of the 2-cell-stage embryo.

Higher-order chromatin organization

Chromatin is extensively remodeled after fertilization (reviewed in Jansz and Torres-Padilla, 2019). Long-range chromatin interactions have been mapped by chromatin conformation capture protocols in both 2-cell embryos (Du et al., 2017; Flyamer et al., 2017; Ke et al., 2017) and 2CLCs (Kruse et al., 2019). These studies revealed that topologically associating domains (TADs) and loops are weaker in 2CLCs than in ESCs. Approximately 1500 genomic regions undergo changes in chromatin structure in 2CLCs, primarily gaining insulating properties and often forming new TAD boundaries. The establishment of 2CLC-specific insulating regions occurs largely at MERVL elements. Thus, MERVL activation in 2CLCs leads to insulation of domains and increased boundary formation at MERVL loci (Kruse et al., 2019). A similar rearrangement of the chromatin architecture occurs at the early 2-cell stage of embryonic development, concomitant with MERVL expression (Kruse et al., 2019; Wu et al., 2016). This evidence further supports the totipotency-specific characteristics of 2CLCs, showing that 2CLCs adopt a chromatin organization reminiscent of that of the 2-cell embryo.

Global DNA hypomethylation

Analysis of DNA methylation in 2CLCs revealed global hypomethylation relative to ESCs grown in serum/LIF (Eckersley-Maslin et al., 2016). Hypomethylation occurs evenly across chromosomes and all genomic features analysed, including gene bodies, promoters, enhancers, and all repeat classes. DNA methylation levels are globally reduced from about ~70% in ESCs to about ~50% in 2CLCs (Eckersley-Maslin et al., 2016). Global DNA methylation levels also decrease in early mouse embryogenesis, from the zygote to 2-cell stage, as the paternal genome

is actively demethylated (reviewed in Messerschmidt et al., 2014). Hence, DNA demethylation seems to be a shared feature between these two reprogramming events - 2CLC reprogramming in vitro and epigenomic reprogramming after fertilization in vivo.

'Active' histone marks

In line with a globally more plastic chromatin, as judged by histone mobility and TAD/loop contact strength, 2CLCs display higher levels of some histone modifications associated with transcriptional activation. For example, Western blot and/or immunostaining analyses indicate that 2CLCs display higher levels of the 'active' marks H3K4me2, pan-acetylated H3 and pan-acetylated H4 compared to ESCs (Ishiuchi et al., 2015; Macfarlan et al., 2012). While global levels of such histone modifications may not be directly compared between embryos and 2CLCs, it is known that H4 is predominantly di-acetylated in zygotes and 2-cell embryos (Wiekowski et al., 1997). Notwithstanding, the role of H3K4 methylation - specifically H3K4me3 - in oocytes, zygotes and 2-cell embryos may differ to that in ESCs and somatic cells, since H3K4me3 is known to form atypical broad domains in mouse oocytes, which are resolved at the 2-cell stage (Dahl et al., 2016; Zhang et al., 2016). The role of H3K4me3 in regulating 2CLC transcription remains to be addressed.

Metabolic state

In addition to the aforementioned transcriptional and chromatin-related features, 2CLCs exhibit a number of distinctive metabolic characteristics. They display lower mitochondrial respiratory capacity and reduced oxygen consumption rates than do ESCs (Rodriguez-Terrones et al., 2020). 2CLCs also produce less reactive oxygen species (ROS) than ESCs grown in serum/LIF conditions, consistent with decreased aerobic respiration rates of 2CLCs (Rodriguez-Terrones et al., 2020). Early embryos also display lower oxygen consumption compared to blastocysts, which has been associated with their smaller and less complex mitochondria (Dumollard et al., 2009). However, while mitochondria of 2-cell embryos are spherical, electron rich and have concentrically organized cristae (Stern et al., 1971), 2CLCs have elongated mitochondria with electron poor matrices and irregularly folded cristae (Rodriguez-Terrones et al., 2020). Thus, similar to early embryos, 2CLCs recapitulate lower oxygen consumption rates, but their mitochondria morphology differs.

Two studies found decreased glycolytic activity in 2CLCs, further supporting a metabolic shift upon the transition of ESCs to 2CLCs (Hu et al., 2020; Rodriguez-Terrones et al., 2020).

Similarly, the 2-cell embryo has low glycolytic activity, low oxygen consumption and relies on pyruvate and lactate as energy sources (Kaneko, 2016; Leese and Barton, 1984; Rodriguez-Terrones et al., 2020). Thus, while more work is needed to fully comprehend the metabolic features of 2CLCs, it appears that 2CLCs recapitulate the low oxygen consumption and the low glycolytic activity of 2-cell stage embryos.

Developmental potential

Currently, we posit that there is no true assay to properly determine the totipotency of cell culture models. To date, the totipotency of embryonic cells has been tested by introducing a single blastomere into an empty zona pellucida, then implanting it back into the oviduct of a mouse (Casser et al., 2017; Tarkowski, 1959). The ability of the blastomere to develop, implant and give birth to viable offspring proves totipotency, according to the stringent definition. However, performing this using a single cultured cell can be technically challenging and, to the best of our knowledge, the approach above has not been used successfully to assess the developmental potential of individual 2CLCs. Several other approaches have been applied (see **Table 2**), although the number of studies assessing 2CLC potential remains low, and the methods used are not always concordant. More studies are therefore necessary to reproduce the presented data and confirm the developmental potential of 2CLCs.

The initial experiment that supported an expanded cell fate potential of 2CLCs used chimera formation analyses upon aggregation of four 2CLCs with morula stage embryos (Macfarlan et al., 2012). Of the five embryos analyzed, three seemed to have 2CLCs in both the ICM and TE at the blastocyst stage. By contrast, of the five control embryos analyzed, which used ESCs for aggregation, ESCs could be detected in the ICM but not the TE in all 5 of them. However, these contributions were analyzed using epifluorescence microscopy, which is not the most accurate of microscopy approaches, since the 3D layout of the blastocyst cannot be properly assessed. Upon blastocyst implantation (not shown in Table 2), immunofluorescence showed that 2CLCs contributed to all embryonic tissues as well as the trophoblast giant cells from the placenta, primordial germ cells, and yolk sac. However, the number of embryos analyzed and the number of cells used was not described, therefore it is difficult to fully appreciate the significance of this data (Macfarlan et al., 2012). Overall, this would suggest that 2CLCs might have a bipotential competency, based on their potential to contribute to both the TE and ICM lineages. It will be important to repeat these experiments in more replicates and using different culture

conditions to control for the effect of the medium on developmental potential, since this has also been shown to alter the ability of ESCs to contribute to the TE (Morgani et al., 2013).

The potential of 2CLCs was later tested using induced 2CLCs. As mentioned above, these cells arise under conditions that increase the frequency of 2CLC reprogramming. The first study showed that ESC cultures deficient for the microRNA, miR34a, contain more induced 2CLCs (Choi et al., 2017). Although the induced 2CLCs arising from miR34a$^{-/-}$ cells were not directly analysed for developmental potential, this study did compare cells that do not express miR34a (*miR34a*$^{-/-}$) with wild type (WT) ESCs. The results obtained using different assays are summarized in **Table 2**. Using teratoma and embryoid body formation assays, it was shown that markers of TE and extra-embryonic endoderm, a derivative of the ICM, are highly expressed in *miR34a*$^{-/-}$ cells relative to WT ESCs. In morula injection experiments, the numbers of chimeric embryos showing contribution of miR34a$^{-/-}$ cells to TE and ICM (indicated by their position in the blastocyst) are in agreement with those numbers reported by the Macfarlan et al. study (i.e. 16/27 miR34a$^{-/-}$ and 3/5 2CLCs). In both studies, no ESCs were found to be localised to the TE or its derivates in chimaera assays (0/7 embryos in Choi et al., 0/5 embryos in Macfarlan et al.). This would support the idea that 2CLCs may have a higher potential to contribute to both ICM and TE-derived lineages.

A more recent study described the maternal factor NELF-A as an inducer of 2CLCs (Hu et al., 2020). The authors used a significantly higher number of blastocysts to assay for the bipotential competency of the resultant induced 2CLCs. Their results suggest that NELF-A 'high' expressing cells have a higher probability of bipotential blastocyst contribution (12 out of 73 embryos), as judged by their position in either the TE or the ICM using confocal microscopy, compared to NELF-A 'low' expressing cells (0 out of 74 embryos) (Hu et al., 2020).

While these chimera assays are used as the gold-standard to test for embryonic pluripotency, they may not be best suited to assess totipotency. First, chimaera assays are performed at a stage at which the cells used as 'recipients' are already beyond the totipotency stage; as such, the microenvironment into which the cells are injected does not recapitulate the environment in which totipotent cells are normally found (i.e. the zygote and 2-cell embryo). This means that cells injected into morula or blastocyst stage embryos would receive cell-cell interaction information and extra-cellular signals that are most probably different from those that totipotent blastomeres receive. Second, teratomas and embryoid bodies from wild type ESCs also express

extra-embryonic markers, although at low levels. Therefore, expression of these markers is not specific to totipotent cells in these assays, and conclusions on totipotency *per se* cannot be drawn from this assay.

In a study from our lab, we used nuclear transfer to test the reprogramming efficiency of 2CLCs as an assay to measure cellular plasticity (Ishiuchi et al., 2015). This experiment cannot determine the potency state of a cell, as somatic cells can also be reprogrammed this way (Gurdon, 1962; Wilmut et al., 1997); however, the reprogramming efficiency does depend on the potential of the donor cell, and accordingly early embryo blastomeres are better donors than ESCs and terminally differentiated cells (McGrath and Solter, 1984). The success rate in reprogramming upon nuclear transfer for both endogenous 2CLCs and CAF1 knock-down induced 2CLCs was 4 and 2.5 times higher, respectively (52/210 and 30/197 transfers), compared to ESCs (Ishiuchi et al., 2015). These data demonstrate that 2CLCs display higher plasticity than ESCs, but they cannot formally inform us of whether or not 2CLCs are in fact totipotent. Thus, a new assay is clearly needed to define whether a cultured cell is totipotent or not.

Concluding remarks and perspectives

With this article, we have aimed to provide the scientific community with a comprehensive list of reproducible features that characterize 2CLCs. While new ways to induce 2CLCs by adding metabolites to the culture medium have been reported (Hu et al., 2020; Rodriguez-Terrones et al., 2020), 2CLCs remain a rare cell type, which poses a challenge to biochemical and molecular investigations. Of note, the 2CLCs are not only rare because they appear at low frequency in mouse ESCs, but also because so far they have been only described in the mouse. Indeed, to the best of our knowledge, a similar metastable phenomenon leading to '2CLCs' or alike has not been described in other species, e.g. humans. It is also important to keep in mind that the percentage of 2CLCs may vary between cell lines and clones, but also between culture conditions (e.g. 2i vs serum/LIF). This is particularly relevant when evaluating the bipotent chimeric competency of 2CLCs, given that ESCs cultured under different culture conditions show different rates of bipotent competency (Martin Gonzalez et al., 2016).

In this light, we would like to suggest caution when referring to 2CLCs. For example, they are often referred to as "totipotent stem cells". However, 2CLCs do not show features of stem cells, or at least they remain to be properly demonstrated. ESCs are called "embryonic" because they

are derived from the embryo, and "stem cells" because of their self-renewal ability in culture and their ability to contribute to all tissues of the embryo proper, including the germline, when assembled into chimeras. 2CLCs might emerge from ESCs, but they are neither embryonically-derived nor 'stem'. There is also no evidence to date to suggest that 2CLCs self-renew. Without appropriate culture conditions to maintain and self-renew 2CLCs, it will be impossible to perform differentiation protocols starting from 2CLCs. However, studies of the mechanisms underlying 2CLC reprogramming are beginning to shed light on how 2CLCs emerge and the pathways that can induce them (Eckersley-Maslin et al., 2016; Fu et al., 2019; Hendrickson et al., 2017; Hu et al., 2020; Ishiuchi et al., 2015; Rodriguez-Terrones et al., 2018; Rodriguez-Terrones et al., 2020). These insights could help establish culture conditions to stabilize 2CLCs in culture, which would be a critical step towards making 2CLCs a robust *in vitro* model of the 2-cell stage embryo. Indeed, the propagation of 2CLCs in culture is a current challenge for the field. In analogy, similar work that led to robust culture conditions for ESCs decades ago will be necessary to figure out the optimal combination of growth factors, medium and molecules that may be needed for 2CLCs to propagate stably in culture.

2CLCs clearly deserve their name, according to the many features they share with their in vivo counterparts, the blastomeres of the 2-cell embryo. However, while it is clear that 2CLCs present us with an opportunity to model and better understand the totipotent 2-cell stage, they are not identical. One of the most important differences concerns the developmental potential of the two cell types: the blastomere of a 2-cell embryo can develop into a full embryo and supportive tissues without the need for carrier cells while, so far, a 2CLC can only contribute to chimeras. This limitation of 2CLCs may stem, for example, from two other major differences between 2CLCs and 2-cell embryos: their maternal transcripts and their size. An in-depth study comparing the maternal transcripts still present in the 2-cell embryo to those present in 2CLCs is lacking, but it is known that the 2-cell embryo contains maternal RNAs that undergo degradation after ZGA, after the 2-cell stage (reviewed in Schier, 2007), as well as maternal proteins. Such RNAs and proteins are likely to be crucial for the successful development of the early embryo and, hence are likely to be crucial for totipotency. However, ESCs derive from the blastocyst, an embryonic stage that lacks the stock of maternal transcripts and proteins, and while 2CLCs reactivate some maternal transcripts (e.g. *Obox*) they do not seem to fully recapitulate the load of maternal transcripts of their in vivo counterparts. Another potentially important difference between 2-cell blastomeres and 2CLCs is their size. The diameter of a one 2-cell blastomere is approximately 80 μm while that of 2CLCs, like ESCs, varies between 8

and 15 μm. This could impact the ability of generating, for example, a blastocyst of the correct size following the early cleavage steps where the size of the embryo does not increase.

Finally, regarding whether 2CLC are indeed totipotent, this depends entirely on how one defines totipotency. Even when using a more relaxed definition of totipotency, no study (to our knowledge) has shown that a single 2CLC can proliferate and colonize both embryonic and extra-embryonic tissues of an embryo when injected into the morula (or earlier stages). Nevertheless, we sustain that there is currently no suitable way to robustly assess totipotency *in vitro*. This will be a cornerstone of research for the field in years to come.

Thus, we propose that 2CLCs should be defined as transient, totipotent-like cells, emerging spontaneously from mouse ESC cultures and sharing specific molecular, chromatin, nuclear organization and metabolic features with totipotent 2-cell stage blastomeres of the mouse embryo. These cells therefore constitute an invaluable model for studying, addressing, and manipulating the molecular features of totipotent cells, by providing a biochemically tractable and more accessible model.

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Box 1. Defining totipotency: one word, several meanings.

In the field of stem cell and developmental biology, the word "totipotency" has been associated with different meanings, as reviewed previously (Baker and Pera, 2018; Condic, 2013; Morgani and Brickman, 2014; Torres-Padilla, 2020 (PTRS accepted)). Two different definitions are widely used, leading to confusion in the field. The broader, perhaps less stringent, definition refers to the ability of a cell to give rise to all extra-embryonic and embryonic tissues, including the germline. Based on this definition, a 4-cell stage blastomere of the mouse would be considered totipotent. In this Spotlight, we use the more stringent definition, and define totipotency as the ability of a single cell to develop into a whole organism by itself. This implies that a cell can develop and form all supportive, extra-embryonic tissues necessary for the embryo to grow, as well as the embryo itself, without the need for carrier cells. This would

anticipate that upon placing a single cell into an empty zona pellucida and subsequently transferring it back *in utero*, a totipotent cell will develop into a new organism. Therefore, while extremely plastic and able to contribute to all tissues of the embryo, blastomeres present later than the 2-cell stage in the mouse are not totipotent.

Box 2. Expanded potential stem cells (EPS cells) versus 2CLCs: false friends.

Extended potential stem cells (EPS cells; Yang et al., 2017) are not the same as 2CLCs (Macfarlan et al., 2012). Their name and properties can lead to confusion because 2CLCs have been described to have 'expanded cell fate potential'. However, even though both types of cells display increased developmental potential and can lead to the birth of viable chimeric pups, they are dramatically different. The most important difference is their transcriptome, as can be visualized in PCA analyses comparing mESCs, Macfarlan 2CLCs and mEPS cells (Yang et al., 2017). While 2CLCs and mESCs are similar at the transcriptomic level, EPS cells clearly distinguish themselves from the others. 2CLCs do not express extra-embryonic markers, but they do have the ability to form extra-embryonic tissues in chimeras. EPS cells, on the other hand, already express markers of embryonic and extra-embryonic lineages when in culture. Finally, EPS cells are actually reprogrammed cells that arise when using the complex 'LCDM' culture cocktail (Yang et al., 2017), which can also be adapted for human cells. By contrast, 2CLCs are spontaneously occurring cells that arise in ESCs cultures grown simply in serum/LIF.

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			2CLC	ES cell
Transcriptional features	Pluripotency markers	OCT4	+/-	+++
		SOX2	+/-	+++
		NANOG	+/-	+++
	2C genes	Zfp352	+++	+
		Eif1a/Eif1a-like	+++	+
		Zscan4	+	-
		Duxf3 (early 2C)	+	-
	Repetitive elements	MERVL	+	-
		MajSat	++	+
Chromatin	Histone mobility	H2A, H3.1	++	+
	Chromocenters	Foci formation	-	++
	ATAC-seq	MT2_Mm and MERVL-int	More accessible	-
		Promoters 2C upregulated genes	More accessible	-
		ES cell specific enhancers (e.g. Prdm14)	Less accessible	-
	DNA methylation	Global	+	++
		MERVL and MajSat	+	++
	Active histone marks	H3K4 methylation	++	+
		panAcH3	++	+
		panAcH4	++	+
Nuclear organization	Hi-C	TADs insulation	+++	+
		Boundary at MERVL loci	+	-
		TADs and loops strength	+	+++
Metabolism	Oxidative metabolism	Glycolytic activity	+	+++
		Oxygen consumption	+	+++
		Pyruvate uptake	-	+
		ROS accumulation	+	+++
	Mitochondria	% elongated (>1.5µm)	25%	9%
		Matrix electron density	Poor	Rich
		Cristae	Irregularly folded	Developed

Table 1 : Known characteristics of 2CLCs.

Table 1 summarizes known features of 2CLCs by comparison with ESC whenever possible. All references are detailed in the discussion. Ratings (+/-) are subjective and aim to visually represent the depth of the differences. In the ATAC-seq part, ESCs are rated "-" as they are the baseline of comparison for 2CLC.

		2CLC		ES cell	
Nuclear transfer	Donor cell	Endogenous	CAF1KD	ES	
	# successful transfer reaching morula/blastocyst	52/210	30/197	12/200	
Chimera assays	Morula microinjection	4 endogenous 2CLCs		4 ES cells	
	# chimeric blastocysts with daughter cells in ICM+TE	3/5		0/5	
	Morula microinjection	1 miR34a$^{-/-}$ cell	4 miR34a$^{-/-}$ cells	1 WT cell	4 WT cells
	# chimeric blastocysts with daughter cells in ICM+TE	13/40	16/27	-	0/12
	Blastocysts microinjection	10-15 miR34a$^{-/-}$ cells		10-15 WT cells	
	# chimeric embryos with daughter cells in TGC or spongiotrophoblast	3/11 E9.5	5/12 E12.5	0/7 E9.5	0/1 E12.5
	#of chimeric embryo with daughter cells in visceral endoderm	4/11 E9.5	2/12 E12.5	0/7 E9.5	0/1 E12.5
	#chimeric embryos with daughter cells in both yolk sac and placental tissues	2/11 E9.5	1/12 E12.5	0/7 E9.5	0/1 E12.5
	Late morula microinjection (E3.25)	5-7 NELF-A "high" cells		5-7 NELF-A "low" cells	
	# chimeric blastocysts with daughter cells in ICM+TE	12/73		0/74	
Teratoma formation	n=3 ; 2 litermate cell lines	miR34a$^{-/-}$ cells		WT	
	TGC-like cells and PL-1 expression	+		-	
	Trophectoderm markers (relative)	+++		+	
	Primitive endoderm markers (relative)	+++		+	
Embryoid body differentiation	n=3 ; littermate cell lines	miR34a$^{-/-}$ EB		WT EB	
	Three germ layers markers (relative)	+		+	
	Trophectoderm markers (relative)	+++		+	
	Extra-embryonic endoderm markers (relative)	+++		+	
	Trophoblast markers (relative)	+++		+	

Table 2 : 2CLCs developmental potential tests.

Table 2 summarizes the results associated to each technique used to test the bipotentiality of 2CLCs. Nuclear transfer data comes from Ishiushi et al.. Chimera formation assays were performed by Macfarlan et al. (morula microinjection from endogenous 2CLCs), Choi et al. (all miR34a$^{-/-}$ numbers are based on the cell line ESC#1 in the paper, since more data was available for this line) and Hu et al. (NELF-A " high" cells). Teratoma formation and embryoid body formation were performed by Choi et al. (miR34a$^{-/-}$ cells). KD, Knock-down; ICM, inner cell mass; TE, trophectoderm; WT, wild type; TGC, trophoblast giant cell; EB, embryoid body.