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DEVELOPMENT AT A GLANCE

An overview of mammalian pluripotency

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ABSTRACT

Mammalian pluripotency is the ability to give rise to all somatic cells as well as the germ cells of an adult mammal. It is a unique feature of embryonic epiblast cells, existing only transiently, as cells pass through early developmental stages. By contrast, pluripotency can be captured and stabilized indefinitely in cell culture and can also be reactivated in differentiated cells via nuclear reprogramming. Pluripotent stem cells (PSCs) are the *in vitro* carriers of pluripotency and they can inhabit discrete pluripotent states depending on the stage at which they were derived and their culture conditions. Here, and in the accompanying poster, we provide a summary of mammalian pluripotency both *in vivo* and *in vitro*, and highlight recent and future applications of PSCs for basic and translational research.

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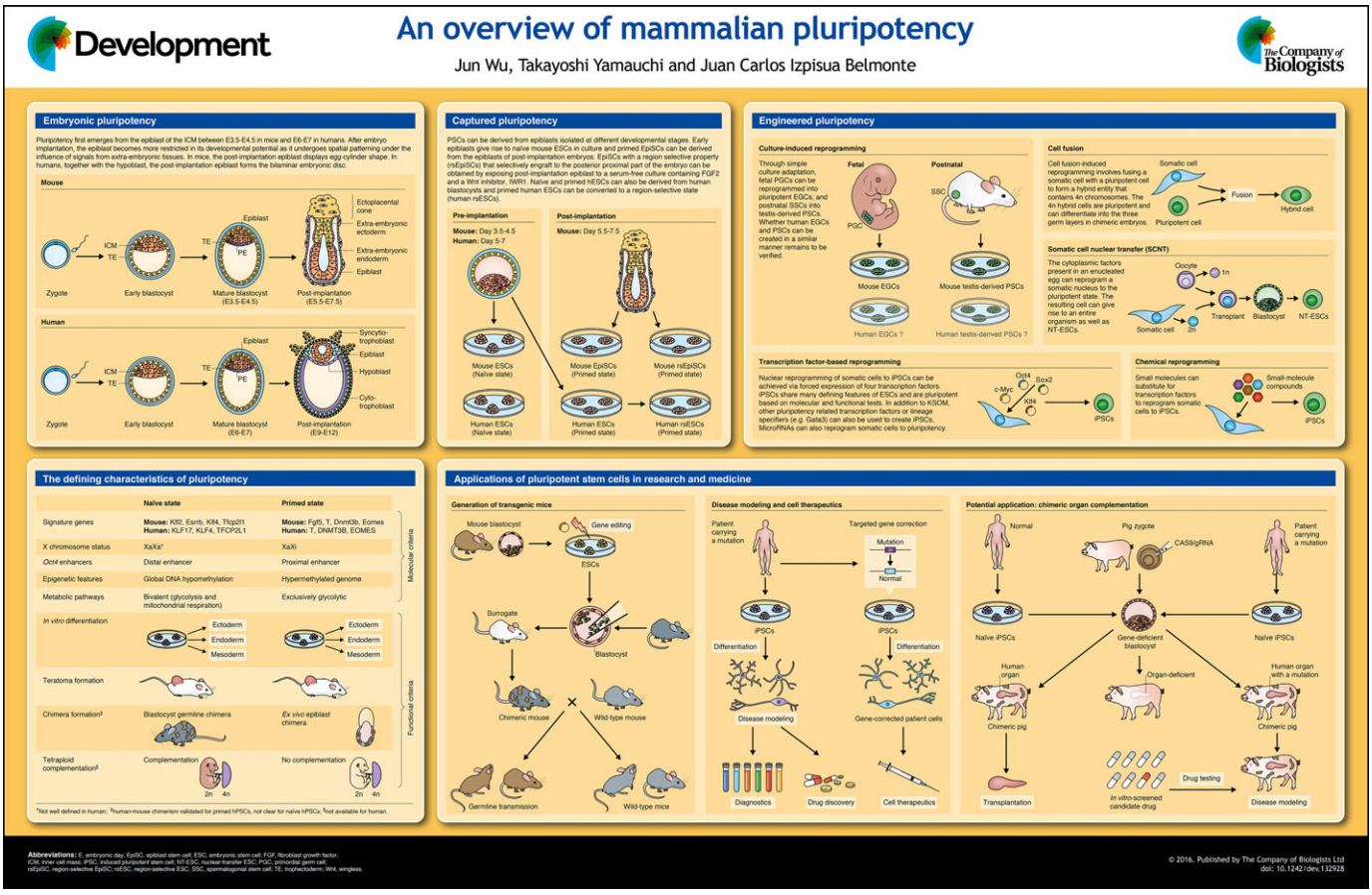
Introduction

Pluripotency is a transient property of embryonic cells during early embryogenesis. It refers to an unrestricted developmental potential to give rise to all three embryonic germ layers – the endoderm, ectoderm and mesoderm – and, eventually, to all cell types that make up an adult organism. Pluripotency can be captured in culture and the resulting cells are known as pluripotent stem cells (PSCs). Unlike their *in vivo* counterparts, PSCs can self-renew indefinitely, thereby producing an unlimited quantity of cells while retaining their pluripotent status. But not all PSCs are created equal: within the broad definition of pluripotency, different pluripotent states exist, each with distinguishable molecular and functional features (Wu and Belmonte, 2016; Wu and Izpisua Belmonte, 2015). Pluripotency can also be reinstated in differentiated cells either via cell fusion, somatic cell nuclear transfer (SCNT), culture-induced reprogramming, transcription factor-based reprogramming or chemical reprogramming. Some of these recent technologies have allowed the generation of patient-specific PSCs, which have



Development (2016) 143, 1644-1648 doi:10.1242/dev.132928

exciting applications for drug discovery and cell replacement therapies. Furthermore, PSCs have revolutionized modern biology by providing a large repertoire of genetic mouse models and, more recently, have provided an unprecedented means to study human developmental processes and disease modeling via directed differentiation into 3D-organised tissues, known as organoids, or the generation of *ex vivo* interspecies chimeric embryos. In this article and in the accompanying poster, we summarize the defining characteristics of pluripotency as they pertain to both the pluripotent cells of the embryo as well as to cultured PSCs. We outline the different ways in which pluripotency can be captured and engineered *in vitro*, and conclude by summarizing some of the current and future applications of PSCs.

Pluripotency *in vivo*

Shortly after the first cell-fate decision to form the inner cell mass (ICM) and the trophectoderm (TE), the ICM starts to segregate into the extraembryonic primitive endoderm (PrE), which eventually gives rise to the visceral and parietal yolk sacs, and the pluripotent naïve epiblast, the founder of all embryonic lineages including the germline. In mice, pluripotency persists in the epiblast for about 4 days. During this time, hatched blastocysts implant into the uterus and epiblast cells increase in number and expand in space. Signals from the surrounding extra-embryonic tissues help to initiate dynamic changes in the pluripotent epiblast cells, preparing them for subsequent lineage commitment during gastrulation. Spatial patterning of the post-implantation embryo yields epiblast cells with distinct properties reflective of where they are located within the epiblast: anterior, posterior, proximal or distal. Heterotopic grafting of epiblast cells from one region to another, however, indicates that the fate of epiblast cells is not fixed and is determined by the local environment, highlighting their highly plastic nature (Beddington, 1982).

In contrast to the wealth of information derived from mouse studies, we know relatively little about how embryonic pluripotency is regulated in humans (Davidson et al., 2015). *In vitro* fertilization provides an accessible way of studying pre-implantation human development until the blastocyst stage. Recent advancements in single-cell analysis have provided insights into the molecular underpinnings of the pluripotent ICM/epiblast in humans. Several differences between human and mouse have been revealed by these studies, for example expression of *Klf2* and *Esrrb* was only observed in mouse ICM, whereas *KLF17* is likely to be a primate-specific pluripotency factor (Blakeley et al., 2015; Boroviak et al., 2015). Post-implantation epiblast development is essentially inaccessible in humans and the only known information is confined to morphological descriptions. Like most other mammals, the human post-implantation epiblast assumes a flat disc epiblast shape, whereas in mouse it has a unique egg-cylinder morphology.

Pluripotency *in vitro*

Embryonic pluripotency has been successfully captured in culture as PSCs (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Mouse ESCs (mESCs) were the first PSCs to be derived from a mammalian species (Evans and Kaufman, 1981; Martin, 1981). mESCs are sourced from pre-implantation epiblast of the ICM and cultured in growth medium containing either leukemia inhibitory factor (LIF)/serum or 2i [dual inhibition of glycogen synthase kinase 3 (GSK3) and extracellular signal-regulated protein kinase (ERK) pathways] (Smith et al., 1988; Ying et al., 2008). Depending on the timing of isolation and extrinsic growth environments, however, PSCs can exist in discrete pluripotent states *in vitro*, and in mouse, two dominant pluripotent states exist: naïve and primed (Nichols and Smith, 2009). mESCs are considered to be in the naïve pluripotent state because they display a molecular signature reminiscent of naïve epiblast of a mature E4.5 blastocyst (Boroviak et al., 2014), and have the ability to form chimeras following blastocyst injection. By contrast, another pluripotent stem cell type called mouse epiblast stem cells (mEpiSCs) can be derived from a broad range of post-implantation epiblast tissues and show features distinct from mESCs (Brons et al., 2007; Tesar et al., 2007). mEpiSCs are believed to exist in a developmentally more advanced pluripotent state, the so-called 'primed' pluripotent state. Primed EpiSCs are not efficient at generating blastocyst germline chimeras; instead, they can broadly engraft into the post-implantation epiblast and form *ex vivo* epiblast chimeric embryos (Huang et al., 2012; Kojima et al., 2014; Wu et al., 2015). The difference in timing between mESCs and mEpiSCs for re-entry embryogenesis following transplantation, as well as their molecular resemblance to *in vivo* tissues, helped to establish the concept that phenotypically and functionally distinct PSCs can be isolated based on temporal differences during early mouse development (Wu and Izpisua Belmonte, 2015). Most recently, two studies have also placed 'spatial' stamps on captured primed pluripotency (Kojima et al., 2014; Wu et al., 2015). Depending on *in vitro* culture environments, post-implantation epiblast cells can acquire unique regional properties and after being transplanted back to a developing embryo, they selectively engraft to a certain region of the epiblast (Wu et al., 2015). Mouse PSC studies have greatly expanded our knowledge of pluripotency and facilitated our understanding of how these highly dynamic early developmental processes occur (Wu and Izpisua Belmonte, 2015).

Human ESCs (hESCs) can also be derived from pre-implantation epiblasts (Reubinoff et al., 2000; Thomson et al., 1998) as in the mouse. However, several lines of evidence indicate that hESCs differ from mESCs and more closely resemble mEpiSCs (De Los Angeles et al., 2015). This has led to the notion that hESCs are primed, akin to mEpiSCs; however, such a comparison might be overly simplistic and it could be more useful to think of hESCs as a case apart. The advantages associated with naïve pluripotency such as high single-cell cloning efficiency, facile genome editing and higher developmental potential have consequently fueled the quest for culture conditions that stabilize hESCs in a more naïve state. The successful derivation of hESCs with naïve features from human blastocysts has been reported recently by several groups (Gafni et al., 2013; Guo et al., 2016; Theunissen et al., 2014; Ware et al., 2014). Despite the success, however, the various culture conditions used to generate naïve hESCs have resulted in an inherent variability in the molecular signatures of the cells, thus making it difficult to compare naïve hESCs and mESCs (Huang et al., 2014). This has been further complicated by difficulties in developing proper *in vivo* functional assays that are able to validate the human naïve state (De Los Angeles et al., 2015; Wu and Izpisua Belmonte, 2015). Therefore, to date, the issue of whether human naïve pluripotency truly exists as it does in the mouse remains unresolved and continued research along this line is needed (Pera, 2014).

Engineered pluripotent stem cells

In addition to *in vivo* pluripotent epiblast cells, PSCs can also be obtained from unipotent germline progenitors/stem cells through culture-induced reprogramming (Jaenisch and Young, 2008). In mice, primordial germ cells (PGCs), the common precursor of oocytes and spermatozoa, are specified from competent epiblast

Development (2016) 143, 1644-1648 doi:10.1242/dev.132928

cells receiving inductive signals from the extra-embryonic ectoderm (Saitou and Yamaji, 2012). Thereafter, PGCs increase in number while migrating through the developing hindgut and finally colonize the emerging gonads, where they initiate differentiation into either oocytes or spermatozoa (Richardson and Lehmann, 2010). Freshly isolated mouse PGCs quickly undergo apoptosis and are refractory to proliferation *in vitro*. When supplemented with growth factors including stem cell factor (SCF), fibroblast growth factor 2 (FGF2) and LIF, mouse PGCs can be converted to pluripotent embryonic germ cells (EGCs) (Matsui et al., 1992; Resnick et al., 1992). In culture, EGCs are morphologically indistinguishable from mESCs. EGCs share many defining characteristics of mESCs and have lost the unipotent potential to only commit to germline development. There are, however, some features of EGCs – for example, the loss of genomic imprinting, which is inherited from the original PGCs – that help to distinguish them from mESCs. The derivation of human EGCs has also been reported (Shamblott et al., 1998). Unlike in mice, however, the human EGCs reported to date are not amenable to long-term culture and cannot form teratomas, which is a critical *in vivo* test of pluripotency. Therefore, a true human counterpart of mouse EGCs has yet to be obtained (Yu and Thomson, 2008). PSCs can also be derived from neonatal and adult mouse testis (Guan et al., 2006; Kanatsu-Shinohara et al., 2004; Ko et al., 2009). PSCs from human testis have also been reported but remain controversial (Conrad et al., 2008, 2014; Ko et al., 2010).

PSCs can also be created by reinstating the pluripotency program in differentiated somatic cells. At least four different strategies have been reported to achieve this goal: cell fusion, SCNT, transcription factor-based reprogramming and chemical reprogramming (Yamanaka and Blau, 2010). In the cell fusion approach, a somatic cell can be fused with an ESC to form a pluripotent hybrid cell (Cowan et al., 2005; Tada et al., 2001). SCNT involves the complete reprogramming of a somatic cell nucleus by maternal factors present in an enucleated oocyte (Tachibana et al., 2013; Wakayama et al., 1998). Transcription factor-based reprogramming to PSCs was first reported in 2006 and since then has become the most commonly used approach for reinstating pluripotency in differentiated somatic cells. PSCs generated by reprogramming factors are called induced pluripotent stem cells, or iPSCs. Initial studies of iPSC generation relied on the forced expression of four transcription factors (Klf4, Sox2, Oct4 and c-Myc) into somatic cells (Aasen et al., 2008; Park et al., 2008; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Since then, multiple different combinations of transcription factors and/or microRNAs have also been used to make iPSCs (reviewed in Hochedlinger and Jaenisch, 2015). The efficiency of iPSC formation is generally low and the mechanism is not entirely clear, although comprehensive molecular characterization of the entire reprogramming process has offered some mechanistic insights (Hussein et al., 2014; Wu and Belmonte, 2014). Importantly, the extrinsic culture environments appear to ultimately determine the pluripotent state of the reprogrammed cells. A case in point is that of EpiSCs, which can be directly induced from fibroblasts thereby bypassing an ESC-like state (Han et al., 2011). Most recently, reprogramming to iPSCs was achieved using only chemical compounds, thus removing the need for transgenes (Hou et al., 2013). Interestingly, iPSCs induced by chemical reprogramming transition through an intermediate extraembryonic endoderm (XEN)-like state, in contrast to transcription factor-mediated reprogramming (Zhao et al., 2015).

How to define pluripotency

The generation of different types of PSCs poses a great challenge with regard to defining pluripotency. This is especially the case in the human system, where there is a lack of *in vivo* studies because of limited access to embryonic tissues. At the molecular level, all PSCs express a set of core pluripotency-related transcription factors that distinguish them from somatic cells (Kim et al., 2008). Indeed, this concept formed the basis of a bioinformatics assay for pluripotency based exclusively on gene expression profiles (Müller et al., 2011). Gene expression profiles can also help to distinguish distinct PSC states. In addition, other molecular features, such as X chromosome status in female cells, differential usage of *Oct4* enhancers and epigenetic features such as DNA and histone methylation also vary between different PSC types (De Los Angeles et al., 2015). Different PSC states can also be set apart by the preferential utilization of metabolic pathways involved in energy production and catabolic and anabolic processes (Sperber et al., 2015; Wu and Belmonte, 2015; Zhou et al., 2012). While molecular and metabolic features are useful, functional tests remain the gold standard for establishing the true developmental potential of PSCs. Several assays with varying degrees of stringency have been established: *in vitro* differentiation and *in vivo* teratoma formation are considered to be the minimal requirement to claim pluripotency. Both assays rely on the ability of PSCs to randomly differentiate into derivatives representative of all three germ lineages. Chimera formation and germline transmission have been traditionally used for testing pluripotency of naïve mESCs. In this regard, primed mEpiSCs are inefficient at chimera formation when using a blastocyst host. However, when mEpiSCs are transplanted into the post-implantation epiblast, they can proliferate and differentiate *in vivo* into three embryonic germ layers (Huang et al., 2012). Thus, post-implantation epiblast chimera formation constitutes a functional test for primed pluripotency. The most stringent pluripotency test is tetraploid complementation, which measures the ability of PSCs to generate an entire adult organism. At present, tetraploid complementation has only been achieved for mouse PSCs. In this assay, electrofusion of a two-cell embryo generates a tetraploid host blastocyst into which donor PSCs are introduced. The current functional gold standard for validating the pluripotency of human PSCs (hPSCs) is the generation of teratomas. As a result of ethical considerations, *in vivo* chimera assays to assess the developmental potency of hPSCs are limited to the use of animal host embryos. Primed hPSCs have been shown to efficiently integrate into the post-implantation mouse epiblast and can go on to generate differentiated progeny (Mascetti and Pedersen, 2016; Wu et al., 2015). However, this *ex vivo* epiblast grafting assay is unlikely to become common practice because of the technical challenges involved. Existing naïve hPSCs have yet to be convincingly demonstrated with regard to their ability to generate interspecies chimeric embryos (Gafni et al., 2013; Theunissen et al., 2014). This leaves open several questions regarding how to define the human naïve state and which animal models are appropriate for *in vivo* testing of the developmental potency of hPSCs (Hackett and Surani, 2014; Wu and Izpisua Belmonte, 2015).

Applications of pluripotent stem cells

There are many avenues for the application of PSCs in basic and translational research. The derivation of germline-competent mESCs, combined with gene targeting, has made possible the introduction of foreign DNA into the mouse genome and enabled the generation of thousands of live transgenic mouse models. These mouse models have proved essential for developmental biology

Development (2016) 143, 1644-1648 doi:10.1242/dev.132928

studies, as well as for the understanding and development of therapeutic interventions for human diseases. With the advent of hPSCs, the field of regenerative medicine has been revitalized (Fox et al., 2014; Robinton and Daley, 2012). The ability of hPSCs to proliferate indefinitely in culture while retaining the capability to differentiate into all adult cell lineages has provided an unprecedented way to study early human development and *in vitro* modeling of human disease, and holds great promise for cell-replacement therapies. To date, many hPSC disease models have been established and they continue to provide valuable information about disease onset and progression, and have facilitated the development of therapeutic strategies (reviewed in Tiscornia et al., 2011). PSC-based cell-replacement therapies are rapidly evolving and applications for the eye, pancreas, neurodegenerative disorders and spinal cord injury are making their way into clinical trials. Initial results from several trials are promising; however, it is still too early for these therapies to be widely adopted in the clinic (Trounson and McDonald, 2015). The potential of naïve hPSCs for formation of interspecies chimera could engender a new wave of *in vivo*-based applications including the generation of human organs in animal hosts via interspecies chimeric complementation, *in vivo* disease modeling and *in vivo* drug screenings, although these applications remain a distant possibility for now (Wu and Belmonte, 2016; Wu and Izpisua Belmonte, 2015). With our ever-increasing understanding of the principles of pluripotency and rapidly evolving fields of genome and epigenome editing, it is foreseeable that novel PSC-based applications will surface in the near future.

Conclusion

Our understanding of pluripotency has been greatly enriched thanks to the successful generation of diverse *in vitro* expandable PSC lines. Within the broad umbrella of pluripotency, different PSC lines represent different states of pluripotency, and we are only just beginning to characterize these at the phenotypic and functional level, especially in the human system. Understanding this heterogeneity is an important part of understanding normal embryonic development and is essential for the future application of PSCs in translational medicine research. Despite the fact that we have accumulated a wealth of information on mammalian pluripotency, it is nonetheless probably just the tip of the iceberg and we still a have long journey ahead of us.

Competing interests

The authors declare no competing or financial interests.

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Development at a Glance

A high-resolution version of the poster is available for downloading in the online version of this article at http://dev.biologists.org/content/143/10/1644/F1.poster.jpg

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