

Snapshots of Pluripotency

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SUMMARY

Pluripotency is a unique developmental state that lays the foundation upon which the entire embryo is built. Pluripotent cells possess the unique capacity to generate, in an exquisitely defined sequence, all the distinct cell types comprising the fetal and adult organism. The discovery of pluripotent stem cells and now the ability to generate them from differentiated cells has had a profound impact on a vast array of scientific disciplines. In addition to their clinical potential as a source of therapeutic cell types, pluripotent stem cells provide scalable access to otherwise experimentally inaccessible development- and disease-associated biology. Here I provide my perspective on the continuum of pluripotency in the early mammalian embryo. I also discuss how novel genomic technologies are now enabling the capture of molecular “snapshots” of the several distinct pluripotent states that stem cells undergo during this pivotal developmental period.

The Continuum of Pluripotency in the Mouse Embryo

The development of placental mammals is unique in that embryos are nourished by interfacing with the mothers' reproductive tract, in contrast to embryos of other classes of vertebrates that develop outside the womb. Thus, mammalian embryos must generate extra-embryonic cell types to mediate their implantation into the uterus, while at the same time maintain a distinct population of unspecified pluripotent cells to form the embryo proper. To do so, within 5 days of fertilization, the mouse zygote partitions itself into three separate cell populations, the trophoectoderm, primitive endoderm, and epiblast, which carry out these diverse tasks in concert. The developmental potential of each of these cell populations was defined by seminal experiments in which chimeric embryos were generated by cell transplantation into host blastocysts (Gardner, 1968; Gardner and Rossant, 1979; Rossant et al., 1978). These studies, among others, showed conclusively that trophoectoderm cells form the bulk of the fetal portion of the placenta, primitive endoderm cells generate the parietal and visceral yolk sac endoderm, and epiblast cells generate the entire embryo proper as well as additional extra-embryonic tissues such as the amnion and allantois.

Several pioneering studies have shown that epiblast cells in the pre- and post-implantation epiblast function to maintain pluripotency until the onset of gastrulation. During implantation, the trophoectoderm invades the maternal uterine tissue to provide sustained access to nutrient and waste exchange for the remainder of gestation. At this point, the

post-implantation epiblast changes from a small cluster of cells into a pseudostratified epithelium that must remain unspecified while it prepares to differentiate into all of the early somatic and germ cell fates that appear during gastrulation. Evidence that the post-implantation epiblast is capable of generating cell fates from each of the three primary germ layers was provided by experiments in which it was transplanted to ectopic sites in adult mice (Diwan and Stevens, 1976). These data were later supported by fate-mapping studies revealing that individual cells of the post-implantation epiblast were not lineage restricted and could contribute to all three germ layers, even when transplanted from one spatial region of the post-implantation epiblast to another (Lawson et al., 1991; Tam and Zhou, 1996).

Historically, pluripotency was considered a single state, yet it was clear quite early on that epiblast cells before and after implantation were morphologically and functionally dissimilar. In contrast to cells of the pre-implantation epiblast, cells of the post-implantation epiblast did not readily incorporate back into host blastocysts or contribute to the developing embryo in standard chimera assays (Gardner et al., 1985). In retrospect, this observation demonstrated a clear developmental distinction between pre- and post-implantation epiblast cells and provided the first indication that more than one shade of pluripotency might exist.

The Two Dominant Pluripotent Attractor States

It only became possible to study pluripotency and its properties when, in 1981, two groups concurrently reported that they had derived pluripotent cells from mouse pre-implantation blastocyst stage embryos, and that these cells could be expanded indefinitely in culture in an undifferentiated state (Evans and Kaufman, 1981; Martin, 1981). Remarkably, these mouse embryonic stem cells (mESCs), later shown to originate from the pre-implantation epiblast (Brook and Gardner, 1997), could be induced to differentiate into a plethora of functional cell types spanning all three germ layers. Later, stringent *in vivo* assays confirmed the pluripotency of mESCs by showing that when injected into host blastocysts, they integrated into the developing embryo and contributed to all cell types of the resulting chimeric mice, including the germline (Bradley et al., 1984; Nagy et al., 1993). Since then, pluripotent stem cell lines have been derived from earlier



Paul Tesar received his undergraduate degree from Case Western Reserve University (CWRU) in Cleveland, Ohio, in 2003 and went on to earn his D.Phil. from the University of Oxford as a recipient of a prestigious scholarship from the NIH. During his graduate and postdoctoral studies, under the tutelage of Professor Sir Richard Gardner and Dr. Ron McKay, Paul investigated how to harness stem cells as a tool to understand early mammalian development. Paul's early studies culminated in a landmark paper in *Nature* in which he described the isolation of a new type of pluripotent stem cell termed epiblast stem cells. Paul returned home to join the CWRU School of Medicine faculty in 2010 and his laboratory has continued to develop cutting-edge stem cell technologies for better understanding and treatment of nervous system disorders including multiple sclerosis, pediatric leukodystrophies, and brain cancer. Paul is currently an Associate Professor and the Dr. Donald and Ruth Weber Goodman Professor of Innovative Therapeutics at CWRU and a Robertson Investigator of the New York Stem Cell Foundation. In 2015, Paul was honored with the International Society for Stem Cell Research Outstanding Young Investigator Award at the annual meeting in Stockholm, Sweden.

blastomere and morula stage mouse embryos, as well as primordial germ cells (Matsui et al., 1992; Resnick et al., 1992; Tesar, 2005). Later, in a series of groundbreaking studies, it was shown that even adult somatic cells can be “reprogrammed” to a pluripotent state by forcing expression of what are now known as core pluripotency transcription factors, yielding induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Pluripotent cells derived from each of these methods are subtly different in terms of their epigenome, yet remarkably, they show almost identical functional properties. Collectively, these studies support the notion that a single pluripotent state, referred to as naive pluripotency, can

be captured in vitro from mouse embryos or reprogrammed from mouse somatic and germ cells.

In the meantime, pivotal advances were being made in defining pluripotency in human cells. In 1998, human ESCs (hESCs) were isolated for the first time from human blastocyst stage embryos and, like their mouse counterparts, could be renewed indefinitely in culture while maintaining their pluripotent state (Thomson et al., 1998). This achievement marked the beginning of the path for advancing pluripotent stem cells into the clinic for regenerative medicine. In a remarkably short time, this goal has been realized, with a number of clinical trials using hESCs to regenerate damaged or diseased tissues and organs now underway.

Yet despite being derived from identical blastocyst stages, hESCs were perplexingly distinct from their murine counterparts in their morphology, molecular profiles, and their need for different signaling molecules to maintain them in an undifferentiated state (Daheron et al., 2004; James et al., 2005; Smith et al., 1988; Vallier et al., 2005; Ying et al., 2003, 2008). Initially, these differences were largely overlooked and attributed to minor species-specific variation, as opposed to reflecting true significance in the inherent developmental origin and capacity of each cell type.

Then, in 2007, two studies reporting a new type of mouse stem cell type transformed the understanding of pluripotency (Brons et al., 2007; Tesar et al., 2007). The cells, termed epiblast stem cells (EpiSCs), were initially isolated from early post-implantation mouse and rat embryos just prior to gastrulation. In contrast, mESCs characterized in earlier studies were derived from pre-implantation embryos. However, although mouse EpiSCs showed striking similarity to native post-implantation epiblast cells, they did not readily incorporate into the developmentally earlier blastocyst in mouse chimeric embryos. Yet EpiSCs were clearly pluripotent, as demonstrated by in vitro differentiation, teratoma generation, and transplantation into the peri-gastrulation epiblast of in vitro cultured whole mouse embryos (Brons et al., 2007; Huang et al., 2012; Tesar et al., 2007).

These data led to a major shift in how pluripotency is defined. What had previously been defined as the pluripotent state based on the earlier mouse ESC studies represented only a common attractor state inherent to pluripotent cells derived from the mouse pre-implantation epiblast, referred to as naive pluripotency (Nichols and Smith, 2009). EpiSCs, on the other hand, represent a distinct pluripotent state, referred to as primed pluripotency, based on morphological, molecular, and functional criteria. Most strikingly, EpiSCs derived from mice shared defining properties with hESCs. This observation led to the current understanding that standard hESC lines



represent a later stage in development than the blastocyst embryos from which they are derived.

Once it was recognized that distinct pluripotent subtypes do exist, the study of epiblast-derived pluripotent stem cells soared. Now there is an exciting body of work showing that EpiSCs represent a previously undefined attractor state of pluripotency. To date, EpiSCs have been derived from a spectrum of post-implantation stages up through the early phase of gastrulation (Bernemann et al., 2011; Han et al., 2010; Kojima et al., 2014; Wu et al., 2015). In addition, induced EpiSCs (iEpiSCs) have been derived by reprogramming somatic cells with core pluripotency transcription factors in culture conditions distinct from iPSC derivation (Han et al., 2011). Surprisingly, by controlling the culture environment, EpiSCs could even be isolated from pre-implantation mouse blastocysts, which strongly supports the notion that what are currently defined as hESCs progress in culture to this second pluripotent attractor state representative of the post-implantation epiblast (Hanna et al., 2009; Najm et al., 2011).

Why mammals in particular evolved to have more than one pluripotent state during embryogenesis remains an open question. Other vertebrates such as fish and frogs have what appears to be a single pluripotent state akin to the mouse post-implantation epiblast just prior to gastrulation. While purely speculative, it is tempting to consider that the pre-implantation pluripotent state evolved as a mammalian-specific trait to ensure fidelity of the pluripotent cells until a sustained source of nourishment upon implantation in the uterus could be acquired. In line with this idea, many mammals are known to be able to delay implantation of embryos and maintain them in a dormant state, termed embryonic diapause, until favorable conditions are present that enhance the survival of the mother and offspring (Renfree and Shaw, 2000). Upon implantation, the pluripotent cells then transition from a naive state of cellular fidelity to a primed state in which the cells are prepared to rapidly respond to developmental cues and transition into the full complement of somatic and germ cell fates during gastrulation.

Importance of Naive and Primed Pluripotent Stem Cells

The array of pluripotent stem cell lines derived directly from mouse and human blastocysts and through reprogramming provides a scalable source of differentiated cells representing the full spectrum of cell fates, embryonic and extra-embryonic. The ability to readily derive these cells from healthy and diseased mice and humans is transforming cell-based regenerative medicine and providing unprecedented tools for modeling and developing drugs for a variety of previously intractable, incurable chronic diseases. Thus, a pressing question for the field is whether

naive or primed pluripotent stem cells are better suited to these tasks and for use as experimental tools for understanding the mechanistic basis for pluripotency and differentiation.

All pluripotent stem cell lines are capable of providing a source of scalable somatic cell fates for studying their biology, and for disease modeling and cell-based regenerative medicine. Initially, experimenters decided whether to use naive versus primed pluripotent stem cells based on technical advantages that varied depending on the experimental requirements, including the ability to expand them from isolated single cells, their amenability to homologous recombination-based genome editing, their genetic accessibility, and the uniformity of their differentiation response. However, recent advances in culture conditions and nuclease-mediated genome editing have largely obviated technical limitations for both cell types, enabling focus on their true biological differences.

Consequently, the door has now been opened for complementary studies employing both of these distinct pluripotent states, providing unprecedented access to the molecular events occurring at the earliest phases of mammalian development that were not possible by studying naive or primed cells in isolation.

In particular, global epigenetic and transcriptome comparisons between mESCs and mEpiSCs have revealed new insights into naive and primed pluripotency including the molecular mechanisms that maintain pluripotent cells in an undifferentiated state while remaining permissive to differentiation into all somatic and germ cell lineages. While these two pluripotent states exhibit a relatively small number of differentially expressed genes, they differ substantially in the organization of their chromatin landscape, particularly with respect to covalent histone modifications of *cis*-regulatory elements such as enhancers, DNA methylation, and the binding pattern of pluripotency transcription factors (Buecker et al., 2014; Factor et al., 2014). Surprisingly, in each of the two cell states, mESCs and EpiSCs, genes with the same levels of expression, including core pluripotency factors such as Oct4, are controlled by distinct enhancer elements (Factor et al., 2014; Tesar et al., 2007; Yeom et al., 1996). Moreover, naive pluripotent cells such as mESCs do not require expression of any of the catalytically active DNA methyltransferases, whereas primed cells show a clear dependence on proper establishment and maintenance of DNA methylation (Liao et al., 2015). Collectively, these global comparisons suggest that the transition from naive to primed pluripotency is predominantly controlled by changes in chromatin organization. Initially, in naive pluripotent cells, the chromatin is organized in such a way that it safeguards the cells from inappropriate differentiation, maintaining them in a pluripotent state. Then as the cells become primed, the



chromatin switches to an organization that enables the cells to transition to a somatic regulatory program during gastrulation.

Our understanding of the mechanisms controlling additional developmental events in the early mammalian embryo has been uniquely advanced by the combined utility of naive and primed pluripotent stem cell biology. For example, there exists a narrow window in the early post-implantation embryo when a handful of founder primordial germ cells (PGCs) are specified from the epiblast (Hayashi et al., 2007). In the past, attempts to generate PGCs directly from naive and primed pluripotent stem cells yielded limited success. But recently, by leveraging understanding of the two pluripotent stem cells states, groups have now robustly generated mouse and human primordial germ cell-like cells in vitro from cells in a transitional state between naive and primed termed epiblast-like cells (EpiLCs) (Hayashi et al., 2007; Irie et al., 2015). In addition, comparison of female naive mESCs and primed EpiSCs has enabled new understanding of the initiation and maintenance of X chromosome inactivation in female somatic cells (Gayen et al., 2015).

Conclusions

The two dominant pluripotent stem cells states have provided powerful in vitro snapshots of the mammalian pluripotency continuum. Utilization of these in vitro cell states provides unprecedented access to fundamental developmental events that have previously been inaccessible to large-scale molecular analyses. Although we have already seen pluripotent stem cell studies yield two Nobel Prizes, we have likely only scratched the surface of what pluripotent stem cell biology will yield in terms of fundamental understanding of development and disease as well as clinical applications.

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