

A germline-centric view of cell fate commitment, reprogramming and immortality

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Summary

To ensure species continuity, the tantalising developmental plasticity of early embryonic cells, also called totipotency, must be transmitted to the offspring. This responsibility rests within the reproductive cell lineage: the germ line. At the recent EMBO/EMBL symposium ‘Germline – Immortality through Totipotency’, researchers discussed the mechanisms that establish and control totipotency, with an eye towards the mechanisms that may endow germ cells with the ability to propagate totipotency across generations.

Key words: Epigenetics, Germline, Inheritance, Reprogramming, Totipotency

Introduction

The molecular mechanisms underlying cell fate commitment and reprogramming are at the heart of all developmental decisions. Arguably, the most spectacular reprogramming event of all takes place during germ cell formation, which is why germ cells and their various pluripotent derivatives have been invaluable for dissecting the mechanisms controlling totipotency. In addition, re-launching totipotency is crucial for the life cycle to continue. Uniquely focusing on these key aspects of germline biology, Edith Heard, Ruth Lehmann and Janet Rossant organised an enthralling EMBO/EMBL symposium entitled ‘Germline – Immortality through Totipotency’, which was held in Heidelberg in October 2012. The meeting, hosted by the EMBL Advanced Training Centre in the woods above Heidelberg (which, notably, were dressed in autumn colours), had a great opening: only days after being awarded the 2012 Nobel Prize in Physiology or Medicine for the discovery that mature cells can be reprogrammed to totipotency, John Gurdon (The Gurdon Institute, Cambridge, UK) introduced key questions in the reprogramming field, offered a historical perspective and shared some of his new findings. Four major concepts for our understanding of reprogramming were introduced. The first relates to the stability of cell differentiation; indeed, the differentiation state of a cell is remarkably stable because cells, as opposed to nuclei, remain strongly committed to their lineage even in a foreign environment. The second concept refers to the reversal of cell differentiation, which can be appreciated upon nuclear transplantation; it has been established that nuclei change fate relatively well. This indicates that the cytoplasm ‘keeps the nucleus under control’ in terms of its differentiation status. A third concept relevant for nuclear reprogramming is the transcriptional activation of pluripotency genes, which, following chromatin decondensation and histone remodelling (Fig. 1), occurs together with the

repression of unwanted (lineage-specific) genes. Finally, there is the concept of resistance to reprogramming, which is likely to be mediated by proteins that are tightly bound to chromatin, such as the histone variant macroH2A. Overall, it was concluded that reversal to an embryonic gene expression programme during reprogramming depends both on a high concentration of some histone variants, which is a non-selective process, and on a transcription apparatus, which is a selective event. As we highlight below, the concepts introduced by John Gurdon provided a framework for subsequent discussion during the meeting on how totipotency and pluripotency are initially programmed, restricted in development, re-gained again and controlled during germ cell formation, and, finally, safeguarded from various insults to allow for species propagation.

Programming totipotency during the oocyte-to-embryo transition

Our understanding of when and how gene expression and epigenetic information are remodelled towards totipotency remains incomplete. Tackling this problem, Brad Cairns [University of Utah and Howard Hughes Medical Institute (HHMI), Salt Lake City, USA] examined chromatin and DNA methylation in gametes and at four different time points during early zebrafish embryo development. Bulk DNA methylation is high in the sperm, lower in the egg and in 2- and 16-cell-stage embryos, and then rises before zygotic genome activation. Reprogramming occurs in two phases: the majority of transcription start sites for developmental factors are reprogrammed in the germline, and the remainder during the early developmental phases, with parental bias evident. The DNA methylation patterns define four different types of transcription start sites: (1) those that are methylated in the egg; (2) those that are demethylated in the egg; (3) those that become hypermethylated later during development; and (4) those that become hypomethylated during early development. Following on from this, Antoine Peters (Friedrich Miescher Institute, Basel, Switzerland) further delineated an epigenomic view of the molecular determinants of histone retention in mammalian spermatozoa. Peters and colleagues observe that sequence composition is highly predictive for nucleosome enrichment. Furthermore, their data suggest specific functions for canonical and replacement histone H3 proteins in chromatin remodelling during spermiogenesis.

Epigenomic analysis of the mammalian female germline has proven to be difficult technically, but Gavin Kelsey (The Babraham Institute, Cambridge, UK) has made a big step towards this goal. By studying DNA methylation globally in oocytes at different stages of folliculogenesis, he is capturing when different loci acquire methylation. Analyses of a specific locus, *Zac10*, suggest that promoter use determines the extent of DNA methylation over gene bodies. Transcription would thus be a key driver of the establishment of DNA methylation patterns in female germ cells. DNA methylation patterns in oocytes hint at the possibility of

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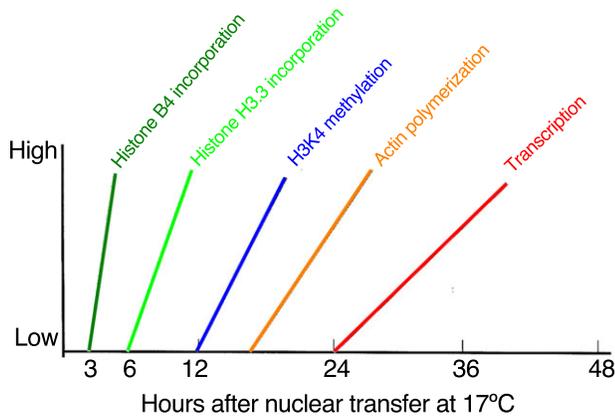


Fig. 1. Time course of transcriptional activation of somatic cell nuclei by oocytes. An approximate estimate of the steps required for transcriptional activation is depicted in the model. Figure kindly provided by J. Gurdon and J. Jullien (both of The Gurdon Institute, Cambridge, UK).

maternal inheritance and, therefore, the potential for transgenerational inheritance. In relation to this, Déborah Bourc'his (Institut Curie, Paris, France) proposed that there are three types of oocyte-specific DNA methylation inheritance: transient, tissue specific and ubiquitous. The ubiquitous type is seen mainly in the case of known maternal imprinted control regions (Proudhon et al., 2012).

How does the embryo interpret the information inherited from the germline during the first moments after fertilisation? One of the main questions is how the newly formed embryonic chromatin is structured and how information is read out from such chromatin organisation during the early stages of embryonic development. Geneviève Almouzni (Institut Curie, Paris, France) described a prominent reorganisation of major satellite sequences (repetitions of a particular short DNA sequence) in early mouse embryos and asked whether expression from these sequences can contribute to chromatin reorganisation. By combining EdU (5-ethynyl-2'-deoxyuridine) incorporation to mark replication with Ring1b and H3K9me3 staining to identify parental origin, Almouzni and colleagues have been examining satellite sequence expression for potential cell cycle and parental bias. Almouzni further discussed how non-coding RNAs might contribute to large-scale chromatin rearrangements and compensate for differences in heterochromatin organisation due to different parental origin. How this epigenetic asymmetry in the parental genomes at fertilisation impacts further development and whether such asymmetry has any role in lineage restriction in the early embryo remains to be determined.

Cell fate commitment and reprogramming

Janet Rossant (The Hospital for Sick Children, Toronto, Canada) asked how the transition from totipotency to pluripotency happens, focusing on the mammalian blastocyst. The early blastocyst comprises two lineages, the inner cell mass (ICM) and the trophoblast (TE), but as development proceeds, the ICM gives rise to two further lineages, the pluripotent epiblast and the primitive endoderm. Therefore, the late blastocyst contains three committed lineages. Rossant defined pluripotency as the ability to block the production of extra-embryonic cell types. When investigating how pluripotent cells are set aside, she concentrated on the first cell fate decision in the blastocyst, during which time the transcription factor CDX2 becomes restricted to the

trophoblast; this occurs before any restriction of ICM markers, which takes place later. Blocking apical polarity through the inhibition of Rho-associated kinase (ROCK) prevents *Cdx2* expression, and the resulting embryo has only 'inside' cells. Apical polarity is required to block the Hippo pathway – a kinase signalling pathway upstream of CDX2 that converges on the transcriptional regulator Yes-associated protein (YAP) – and therefore is required to delineate the first two lineages of the mammalian embryo. In the future, it will be important to define how apical polarity inhibits *Cdx2* expression. As also highlighted by Hiroshi Sasaki (Kumamoto University, Japan), the position of the cell in the early embryo thus plays a key role in specifying embryonic lineages, and the Hippo pathway is a key transducer of such positional information. Indeed, although signalling pathways are expected to be in place, perhaps the physical properties of the cells also have a role in this process; a new avenue of investigations with a physics interface is likely to provide answers to these questions.

Along these lines, Takashi Hiragi (European Molecular Biology Laboratory, Heidelberg, Germany) presented data on the molecular dynamics of lineage segregation. Following a combination of single cell and molecular analyses using Venus-trap lines, he proposed a model for the formation of the pluripotent compartment of the mouse embryo. He suggested that a first step of 'stochastic' onset of gene expression could be subsequently reinforced and adjusted by a combination of multiple signals. Together, they would result in the distinction of the first two lineages of the mammalian embryo. Whether the molecular players underlying lineage restriction in mice are conserved in all mammals was addressed by Stephen Frankenberg (University of Melbourne, Australia). Frankenberg and colleagues have been studying the specification of the pluriblast (the equivalent of the ICM) and the trophoblast in a marsupial, the tammar wallaby. Marsupial (and monotreme) genomes contain two POU-domain proteins related to mouse POU5F1 (OCT4): POU2 and POU5F1. POU2 is a better marker of the pluriblast than POU5F1. Marsupial early blastocysts do not have inner and outer cells, but they do have conceptus polarity with embryonic and abembryonic poles. Neither POU5F1 or POU2 nor the downstream Hippo effectors YAP and WWTR1 are able to distinguish two populations at early stages, and there is no evidence of specification of lineages before diapause. Only after the epiblast emerges do these factors become differentially localised. Thus, although there might be some conservation in the molecular players involved in lineage segregation, the developmental timing of their action might differ.

X chromosome inactivation is a key process that is studied in order to understand better how memory states of transcriptional activity are set apart, how they are transmitted and how they resist changes in cell fate. Edith Heard (Institut Curie, Paris, France) provided evidence that the process of X inactivation is dynamic and shows a great deal more heterogeneity during development than was previously thought. Heard is interested in the kinetics of X chromosome gene silencing and, by assessing the transcriptional status of a number of genes during development using RNA fluorescence *in situ* hybridisation (FISH), her group has uncovered a diversity of situations that depend on the gene and on the embryonic stage and lineage. It is known that the paternal X chromosome is inactivated during preimplantation development but becomes reactivated in the pre-epiblast cells of the ICM of the blastocyst. Heard reported that there might in fact be a flipping of activity states for the paternal X chromosome during the segregation of the epiblast and the primitive endoderm in the ICM

of the blastocyst. Subsequently, random X inactivation is established in the epiblast, around embryonic day (E) 5.5-6.0. By using RNA FISH, her group has recently examined the stability of the inactive X state from E6.5 to E8.0 and found that, although the inactive X is robustly silent in embryonic lineages, trophoblast lineages show up to 40% of cells that escape from X inactivation for some genes at this stage. Trophoblast giant cells, in particular, display epigenetic instability or perhaps plasticity, which might be linked to a rather unusual chromatin configuration of the X chromosome in these cells.

Re-launching the 'immortal' germ line

Azim Surani (The Gurdon Institute, Cambridge, UK) focused on how mammalian germ cells are programmed for totipotency. Surani discussed the molecular mechanisms that underlie primordial germ cell (PGC) specification, which involves setting aside PGCs from the soma and represents a fundamental developmental decision. This decision involves repression of unwanted somatic gene expression, which initiates a genetic programme for epigenetic reprogramming orchestrated by the transcription factors PRDM1, PRDM14 and AP2 γ . This 'tripartite' transcription factor (TF) network for PGC specification lies at the heart of an epigenetic programme that includes parallel redundant mechanisms to reprogramme early PGCs into the basal epigenomic state. A wave of DNA demethylation commences following PGC specification. Surani and colleagues have now shown that this also includes a significant conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) by the enzymes TET1 and TET2. The levels of 5hmC peak at the E10.5 stage. Genome-wide methylated DNA immunoprecipitation followed by sequencing (meDIP-seq) was performed for E10.5-13.5 stage PGCs, and the specific analysis of the *Dazl* locus, for example, by glucosyltransferase-qPCR showed that promoter DNA demethylation is coupled to the acquisition of 5hmC. Overall, Surani pointed out that there is not one single mechanism accounting for epigenetic programming in the germline; the system has to be robust and several parallel pathways contribute to it. Continuing the theme of PGC specification, Mitinori Saitou (Kyoto University, Japan) introduced an *in vitro* system for the production of primordial germ cell-like cells from embryonic stem cells (ESCs) (Hayashi et al., 2011) and described how this approach can be used to evaluate the role of TFs during PGC specification.

Although yeast might seem an unlikely model for studying germ cells, they can, upon encountering adverse environmental conditions, initiate gametogenesis (sporulation) to produce spores. Angelika Amon (Massachusetts Institute of Technology and HHMI, USA) described an elegant mechanism controlling the expression of a key sporulation gene, *inducer of meiosis 1 (IME1)*. In vegetative yeast cells, *IME1* is repressed by a non-coding RNA that is transcribed from the *IME1* promoter. Interestingly, expression of this RNA is linked to recruitment of the Set2 histone methyltransferase and the Set3 histone deacetylase complex to establish repressive chromatin at the *IME1* promoter (van Werven et al., 2012).

Addressing the epigenetic landscape of germ cells, Huck-Hui Ng (Genome Institute, Singapore) reported the identification of factors required for human ESC maintenance in a whole-genome RNAi screen. Among others, this screen identified PRDM14, which 'colocalises' extensively with the H3K27me3 mark on chromatin, associates with the Polycomb group protein EZH2 and represses lineage-specific genes. In relation to the question of how pluripotency is restricted *in vivo*, Ng additionally described a

chromatin immunoprecipitation (ChIP) approach that was optimised to map histone modifications in germ cells isolated from murine testes at the time at which they lose their pluripotent characteristics (~E13.5). Several histone modifications were analysed: H3K4me3, H3K27me3, H3K27ac and H2BK20ac. This study revealed a number of putative enhancers, promoters and transcription factor-binding elements. Additionally, this approach revealed unusual signatures of histone modifications, such as the accumulation of H3K27me3 over whole genes and gene clusters. Consistent with a major role for H3K27me3 in cell fate maintenance, Jacob Hanna (Weizmann Institute, Rehovot, Israel) described the function of UTX (also known as KDM6A) in the efficient reprogramming of mammalian somatic cells and during PGC specification (Mansour et al., 2012). UTX is a JmjC domain-containing enzyme that mediates demethylation of H3K27 tri- and dimethyl repressive chromatin marks. Efficient reprogramming requires the demethylase activity of UTX and, consistently, is enhanced by the inhibition of H3K27 methylation upon the depletion of polycomb repressive complex 2 (PRC2). UTX is constitutively expressed but associates directly with several pluripotency TFs, suggesting a possible mechanism by which its activity is targeted towards specific genes. Along the same lines, Baris Tursun (Max Delbrück Center, Berlin, Germany) reported that the removal of PRC2 could facilitate the reprogramming of *Caenorhabditis elegans* germ cells into somatic cell types upon forced expression of TFs that normally promote somatic fates during embryogenesis (Patel et al., 2012). This study indicates that PRC2-dependent repressive chromatin creates an epigenetic barrier to reprogramming in this model organism as well.

Studying gene expression, cell division and chromatin organisation at the single cell level will strongly advance our knowledge of how the germline is shaped through reprogramming to establish totipotency. For example, using a newly developed light sheet microscope (selective plane illumination microscope or SPIM), Jan Ellenberg (European Molecular Biology Laboratory, Heidelberg, Germany) presented amazing results that address the bi-orientation of chromosomes in mammalian oocytes and the dynamics of chromosome segregation in the preimplantation mouse embryo. In addition, Maria-Elena Torres-Padilla (Institute of Genetics and Molecular and Cellular Biology, Strasbourg, France) has been using a microfluidics approach to address changes in epigenetic components in single cells during early development. These types of approaches will undoubtedly allow analysis at a high temporal resolution to dissect the molecular dynamics of the germline and early embryos and to determine how germ cells transit between reprogramming and totipotency.

Self-renewal versus differentiation during gametogenesis

Once germ cell precursors are specified and colonise the gonad, their proliferation and differentiation must be carefully balanced to prevent stem cell loss or germline cancers. A number of signalling pathways are known to control this balancing act. In this context, Peter Koopman (The University of Queensland, Brisbane, Australia) described a novel function for Nodal signalling in promoting the pluripotency of germ cells in the murine foetal testis (Spiller et al., 2012). This function of Nodal is 'gated' by fibroblast growth factor 9 (FGF9)-dependent expression of the Nodal co-receptor Cripto (Tdgf1 – Mouse Genome Informatics) in testicular germ cells. When Nodal signalling is reduced, the number of pluripotent spermatogonial stem cell progenitors decreases, and germ cell differentiation is stimulated. By contrast, high levels of

expression of Nodal and Cripto correlate with the malignancy of human testicular tumours. A major outstanding question is precisely how signalling pathways dynamically remodel the expression patterns of germline genes. In relation to this, Rafal Ciosk (Friedrich Miescher Institute, Basel, Switzerland) reported that, in the *C. elegans* germ line, signalling to chromatin activates the expression of certain genes by antagonising their repression by PRC2. It has been proposed that this epigenetic remodelling may influence the balance between self-renewal and differentiation.

David Page (Massachusetts Institute of Technology and HHMI, Cambridge, USA) focused on the coordination of growth and differentiation with progression through meiosis. In murine foetal ovaries, *Stra8* is required for meiotic initiation. Page reported that surviving STRA8-deficient ovarian germ cells are capable of growth, form follicles, ovulate and can be fertilised, but that this is followed by developmental arrest in the early embryo. Thus, oocyte growth and differentiation appear to be orchestrated by unknown cue(s) that act independently of meiotic initiation. Switching to the male reproductive tract, Martin Matzuk (Baylor College of Medicine, Houston, USA) pointed out many testis-specific genes that potentially offer targets for male contraception. Towards this goal, Matzuk described the inhibition of BRDT, a member of the bromodomain and extraterminal (BET) family, by a small molecule inhibitor. This inhibitor (known as JQ1) prevents BRDT binding to acetylated histone H4, thus reversibly interfering with chromatin remodelling during spermatogenesis and, hence, with sperm production (Matzuk et al., 2012). Speaking about future directions, Matzuk discussed the potential of using small molecule drugs or recombinant proteins for oocyte maturation in assisted reproductive technology.

Germ cells and the fountain of youth

Siblings produced from young or aging parents have similar lifespans, suggesting that germ cells are relatively resistant to degenerative/aging phenomena observed in the somatic cells. What, then, makes germ cells special in this respect? Venturing into epigenetics, Ruth Lehmann (Skirbal Institute and HHMI, New York, USA) introduced a germline-specific defence mechanism against transposable elements, which depends on PIWI proteins and their associated small non-coding RNAs (piRNAs). Lehmann explained that, in the fly germline, piRNAs are produced in so-called pre-cystoblast cells from heterochromatic clusters. These clusters are marked by dSETDB1 (Eggless – FlyBase) methyltransferase-dependent H3K9me3 histone marks (Rangan et al., 2011). Interestingly, the researchers have identified a possible link between repression of Pol II-dependent transcription and the establishment of repressive chromatin during differentiation of the germline stem cells.

Recently, a lot of attention has been given to the ‘epigenetic memory’ that is transmitted across generations. Anne Brunet (Stanford University, USA) described transgenerational inheritance of longevity, which is mediated by the ASH-2/WDR-5/SET-2 protein complex responsible for H3K4me3 histone modifications (Greer et al., 2011). Alterations in gene expression induced by mutations in this complex can be transmitted for several generations and appear to affect a defined set of transcripts. The Brunet laboratory is currently studying the features of H3K4me3 marks at these de-regulated genes.

Simple genetic model organisms have been instrumental in uncovering mechanisms controlling longevity. Following on from the idea of germ cells having rejuvenating effects, Angelika Amon (Massachusetts Institute of Technology and HHMI, USA)

addressed the potential role of gametogenesis-specific factors in re-setting the replicative lifespan of budding yeast (essentially the number of times a yeast cell can divide). Conveniently, yeast produced from spores have similar ‘lifespans’ irrespective of the age of the mother cells. Remarkably, ectopic expression of the yeast meiosis-specific transcription factor Ndt80 (which is ancestral to the p53 protein family) was reported to reset ‘lifespan’ in vegetative cells (Unal et al., 2011). This resetting correlates with a reversal of changes in the nucleolar morphology that have been linked to aging, suggesting a possible connection with ribosome synthesis and/or the cell cycle. Speaking about aging in vegetative (somatic) cells, Brian Kennedy (Buck Institute, Novato, USA) pointed out that there is a significant overlap between the aging pathways uncovered in yeast and nematodes, raising hopes for conservation all the way to humans. One hallmark of aging is the accumulation of protein aggregates that tend to be retained in budding yeast in a mother cell. Kennedy described an approach to identify proteins that are asymmetrically segregated between mothers and daughters. A significant fraction of the identified proteins does indeed affect aging, as depletion of these proteins extends the yeast ‘lifespan’. Some of these factors exhibit functions in ribosome biogenesis or in mitochondria, raising questions about the adverse impact they may have on the cell retaining them. Extending these findings to mammals, Kennedy suggested that inflammatory responses are to be blamed, and presented evidence linking inflammatory signals to aging-related phenomena in aging mouse tissues. Along similar lines, Miguel Coelho from the Tolic-Norrelykke laboratory (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) investigated the accumulation and distribution of protein aggregates in fission yeast, which normally divide symmetrically. Under normal growth conditions, small aggregates were observed to segregate stochastically with no evident connection to aging. However, upon stress, the aggregates fuse into a large aggregate and become asymmetrically segregated to one daughter cell, which can be explained by a stochastic aggregation model. Importantly, cells inheriting the large aggregates exhibit senescence, reinforcing the connection between stress and aging.

Conclusions

The obvious conclusion from this delightful meeting is that germline biology continues to provide invaluable insights into the mechanisms governing cellular reprogramming. One future challenge is to understand the relationship and crosstalk between the various mechanisms proposed to regulate pluri- and totipotency. Another is, as ever, to interrogate the importance of proposed mechanisms *in vivo*, in order to interpret the relevance of diverse epigenetic modifications. Above all, as some of the presentations provocatively evoked, the relationship between germline reprogramming events and heritable traits, which might affect not only fertility but also somatic physiology, remains an essentially uncharted territory that needs to be explored further.

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Competing interests statement

The authors declare no competing financial interests.

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