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Understanding the first steps in embryonic stem cell exit from the pluripotent state

C. John Luckey, MD PhD, Yu Lu, PhD, and Jarrod A. Marto, PhD

Department of Pathology, Brigham and Women's Hospital, Joint Program in Transfusion Medicine, Department of Cancer Biology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts

C. John Luckey: cluckey@partners.org

Abstract

We are interested in understanding how a given cell type, in response to external cues from its environment, makes the decision to differentiate. In the case of mouse embryonic stem cells (mESC), the key external factor that maintains their undifferentiated state is the cytokine leukemia inhibitory factor (LIF). LIF removal causes mESC to exit their pluripotent state and differentiate into more restricted precursors. Though LIF is known to activate multiple different phosphorylation cascades, the mechanisms by which its removal leads to mESC differentiation are not well understood. In order to identify the molecular events that occur upon LIF removal, we developed a set of novel experimental approaches that allowed identification and quantification of global phosphorylation changes that occur when mESC are deprived of LIF. Intriguingly, LIF removal results in the rapid phosphorylation of multiple proteins known to regulate the mESC self-renewal, though the specific function of these phosphorylations is unknown. Our data set the stage for future studies investigating the functional role of these phosphorylation events in mESC. We hypothesize that these unique post-translational modifications help drive the exit of mESC from the pluripotent state. These studies were greatly facilitated by the NBF, whose support in the crucial initiation phase of these studies was invaluable.

Background

Epigenetic control of development

Soon after fertilization, the single cell zygote begins the orderly process of differentiation¹. Zygotes undergo several rounds of cell divisions, and just before implantation organize themselves to form a blastocyst. A blastocyst is fluid filled sphere made up of an outer epithelial layer of trophoblast cells that go on to form the placenta, and an inner layer of clustered cells at one end of the sphere known as the inner cell mass (ICM). Soon after blastocyst formation, the ICM further differentiates into the epiblast and implantation occurs. After implantation, epiblast cells rapidly differentiate into the three primary germ layers: ectoderm, mesoderm and endoderm. These germ layers subsequently give rise to the full variety of different cell types that make up the tissues in our bodies. The ICM and epiblast are often referred to as pluripotent in that they give rise to all the different cell types of an adult, though they do so via a step wise progression through carefully controlled intermediate stages. This progressive differentiation into distinct cellular types from pluripotent through germ layer to more restricted adult cells is the result of the progressive inheritance of a more specific and limited set of genetic options that do not involve changes

Correspondence to: C. John Luckey, cluckey@partners.org.

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in the DNA sequence; commonly referred to as epigenetics². It is important to remember that at the genome level, all of our different cells are the same. What makes one cell different from another is how that fundamental genetic blueprint is specifically restricted, marked up, read and interpreted. Ultimately, each cell type is unique because of the unique set of genetic elements that it transcribes and silences; and its function is the result of the RNAs and proteins derived from this translation. Our research is focused on understanding how external signals ultimately influence epigenetic programs that dictate cell fate decisions.

Pluripotency in a tissue culture dish: ESC and the artificial maintenance of self-renewal

Given the difficulties of working with developing embryos *in vivo*, researchers have developed *in vitro* cell culture systems to study pluripotent cell function. In initial work with mouse blastocysts, researchers were able to culture isolated ICM cells that they referred to as embryonic stem cells (ESC), and demonstrated that mouse ESC (mESC) could be made to self-renew indefinitely³. Formal proof that mESC maintain pluripotency was shown by chimera and teratoma assays. Chimera assays are performed by injecting cultured ESC into normal developing blastocysts and demonstrating that the injected ESC contributed to all normal tissues through chimerism. Teratoma assays involve injecting cultured ESC into immuno-deficient mice and demonstrating that they subsequently develop into tumors that express mature cells of ectodermal, mesodermal, and endodermal origins.

Pluripotency *in vivo* is normally a short-lived developmental state restricted to the blastocyst and epiblast, and optimal maintenance of mESC in culture requires that they be grown on mouse embryonic fibroblast (MEF) feeder cells. Subsequent studies showed that one of the major reasons mESC required MEFs was that MEFs secreted the soluble cytokine leukemia inhibitory factor (LIF)³. In rodents LIF normally facilitates diapause, which is a process by which developing embryos can be placed into a state of suspended animation during times of stress. Once the stress is removed, the rodent stops secreting LIF and the paused embryos are freed to continue in their development. In a sense, mouse ESC represent an *in vitro* culture artifact wherein MEFs and LIF mimic the suspended animation state normally associated with diapause.

Multiple signaling pathways downstream of LIF

LIF signals are transduced via a heterodimer of the specific LIF receptor beta (LIFRb) and the common IL-6 cytokine family member co-receptor (GP130)⁴. LIF/GP130 signaling activates the JAK1 kinase, which subsequently stimulates at least three major pathways, including: (i) phosphorylation of signal transducers and activators of transcription 3 (STAT3) leading to its dimerization and import to the nucleus where it acts as a transcription factor, (ii) activation of the canonical phosphatidylinositol-3 phosphate kinase (PI3K)/thymoma viral proto-oncogene 1 (AKT) pathway, with concomitant inhibition of glycogen synthase kinase 3 beta (GSK) and (iii) activation of extracellular regulated kinases 1 and 2 (ERK1/2) via the classical mitogen activated kinase (MAPK) pathway. Of these three pathways, STAT3 has been demonstrated to be sufficient for maintenance of pluripotency in mESC⁵, and PI3K/AKT further supports pluripotency through inhibition of GSK. Alternatively, signaling through ERK downstream of LIF has been shown to drive differentiation⁶⁻⁸. Thus the same LIF signal leads to both (i) direct transcriptional activation that supports pluripotency via STAT3 transcription factor activity, (ii) indirect support of pluripotency through PI3K/AKT activity, and (iii) indirectly drives differentiation through ERK activation. Though many of the proximal signaling events downstream of LIF have been described, the specific targets of the PI3K/AKT and ERK pathways are largely uncharacterized in mESC.

How do mESC exit the pluripotent state?

While great strides have been made in the last few years in defining the genetic circuitry required for the maintenance of pluripotency⁹⁻¹², we understand much less about how cells decide to exit the pluripotent state and transition from one transcriptional state to another. In the embryo, the pluripotent state is a transient one, and pluripotent cells receive external signals that normally drive them towards more progressively differentiated lineages. In tissue culture, ESC represent our ability to suspend normal cellular differentiation through the addition of exogenous cytokines such as LIF. Given LIF's central role in pluripotency, we investigated the downstream phosphorylation targets of LIF in mESC.

Results and Discussion

In order to understand the earliest molecular events in mESC fate commitment, we set out to survey the dynamics of phosphorylation driven signaling in mESC in response to the removal of the cytokine LIF. We have employed state-of-the-art mass spectrometry-based phospho-proteomics to describe this key developmental cell-fate transition, and have identified the molecular events that occur as mESC exit their pluripotent state. This work is a coordinated and collaborative effort between two laboratories with different and complimentary skill sets; the Marto laboratory's expertise in analytical chemistry and protein biochemistry and the Luckey laboratory's skills in manipulation of mESC and developmental biology.

Biochemical isolation of pluripotent mouse ESC

mESC grow best when cultured on a mouse embryonic fibroblasts (MEF) feeder layer in the presence of exogenously added LIF. We therefore initiated our studies of mESC signaling by examining mESC signaling changes upon LIF removal when they are grown upon MEFs. However, Figure 1a shows that MEFs contributed significantly to detected signal for pTyr705-STAT3, suggesting that our ability to apply quantitative phospho-proteomic techniques to profile changes in mESC signaling would be compromised by MEF contamination. To circumvent this obstacle, we adapted mESC to culture on permeable membrane inserts suspended above feeder cells (Figure 1b). mESC grown under these conditions maintained expression of pluripotency markers (Figure 1c-g) and supported generation of chimeric mice (Figure 1h). We did note that mESC grown on inserts required more careful attention to every other day passaging and daily media changing; suggesting that mESC-MEF cell contact does play a minor role in stabilizing the pluripotent state. However, mESC were able to be maintained as pluripotent lines for over 20 passages on inserts. Of note, unlike gel or matrigel adaptation procedures, growth of mESC lines on inserts above MEFs resulted in sustained pluripotency of the entire line without requiring further passage, adaptation, or selection. Indeed, we reproducibly passaged all mESC lines tested, regardless of genetic background or source. Furthermore, culture on inserts proved much less sensitive to serum (or serum replacement) batch-to-batch variability, and proved robust in chemically defined media as well (data not shown). Collectively this suggests that MEFs support mESC pluripotency largely through secretion of LIF and other soluble factors. Most importantly, culture of mESC on inserts suspended above MEFs allowed us to directly interrogate the changes in signaling that occurred immediately upon enforced differentiation in a variety of conditions and cell lines.

A Robust and Sensitive Phospho-proteomics Platform to Quantify Early Signaling Events in ESC

Having established a culture system that both robustly supported mESC pluripotency and allowed for facile biochemical study of purified mESC, we next needed to develop the instrumentation and workflow for quantitative identification of the diverse set of

phosphorylation events that occur in mESC. In order to selectively purify and label the phospho-peptides in mESC, we adapted our permeable membrane mESC culture system to a workflow in which phospho-peptides are selectively enriched and labeled with a unique isobaric tag ¹³ from mESC grown under different conditions (Figure 2). In this way, phospho-peptides from different conditions are differentially labeled, purified, mixed together and analyzed by mass spectrometry. We have previously reported new methodology for fabrication of miniaturized LC-electrospray assemblies that interface directly with mass spectrometers and provided for efficient quantification of tyrosine phosphorylated peptides from only around 1 million mESC ¹⁴, enabling us to perform these studies from reasonable numbers of cells.

Phospho-tyrosine confirmation of LIF pathway signaling in ESC

In order to test the effectiveness of our experimental approach, we first investigated the changes in phospho-tyrosine signaling that resulted from LIF and MEF removal from pluripotent mESC. We removed both exogenous LIF as well as MEFs to ensure the complete withdrawal of all sources of LIF for mESC. In this situation, the removal of other potential soluble factors secreted by MEFs is unavoidable. However, the net result of MEF co-culture is mESC self-renewal and pluripotency, and the effects of removal of all these secreted factors should provide important insights in mESC transition from a pluripotent state. Tyrosine phosphorylation is known to be an important regulator of many signaling cascades, and LIF signaling induces the phosphorylation of tyrosine 705 on STAT3. mESC were either kept in pluripotent sustaining conditions or grown in the absence of LIF and MEFs for different time points. Proteins were isolated, reduced, and alkylated prior to digestion to peptides with trypsin. The resultant peptide mixture from individual conditions was subsequently labeled with different isobaric tags (Figure 2). Given that phospho-tyrosine represents < 1% of the total protein phosphorylation events in a given cell, we first purified phospho-tyrosine containing peptides using a mixture of phospho-tyrosine specific antibodies ¹⁵. The resultant labeled peptides enriched for phospho-tyrosine modifications were next separated by an extended reverse phase gradient using our ultra-sensitive electrospray apparatus directly coupled to a time of flight mass spectrometer ¹⁴. The time of flight mass spectrometer was run in data dependent mode, and resultant MS/MS scans provided both sequence information as well as quantitative information. Consistent with the known phosphorylation changes induced by LIF signaling, we observed that tyrosine 705 phosphorylation of STAT3 was rapidly reduced upon LIF/MEF removal (Figure 3). We further identified nearly 150 unique phospho-tyrosine containing peptides and observed regulation on 20 different proteins; the majority of which were not previously known to be downstream of LIF (data not shown). We are currently in the process of determining the functional relevance of these novel phosphorylation events in mESC. However, our observation that Tyr-705 of STAT3 was changed by LIF/MEF removal validated our overall experimental approach, and encouraged us to further characterize the other phosphorylation changes induced by LIF/MEF removal.

Identification of novel phospho-serine/threonine targets of LIF signaling in mESC

Given that LIF also acts through both ERK1/2 and AKT/GSK, which are themselves phospho-serine/threonine kinases, we were particularly interested in examining the modulation of serine and threonine phosphorylation downstream of LIF. This provided an entirely new set of technical challenges. Purified phospho-tyrosine containing peptides are relatively restricted in their number and diversity, allowing for mass spectrometric analysis with only minimal separation. The real technical issue is performing the affinity purification. In contrast, phospho-serine/threonine containing peptides pose a different set of problems. First, there are no good antibodies that work well for global immuno-precipitation of phospho-serine/threonine containing peptides. Second, phospho-serine/threonine is

extremely abundant in the cell, and can be found on many more proteins. And so we set out to develop a robust protocol for the isolation, identification, and quantification of the vast numbers of phospho-serine/threonine containing peptides from mESC.

In order to analyze phospho-serine/threonine containing peptides, we first took advantage of recent improvements in charged-metal based affinity chromatography, in which the charge of the phospho-peptides is used to enrich them relative to their non-phosphorylated counterparts¹⁶¹⁷. Phosphorylated peptides from cells grown in the presence or absence of LIF/MEFs were enriched, labeled with isobaric tags and then mixed. This approach creates an embarrassment of riches, resulting in thousands of different phospho-peptides purified from each condition. To address the level of complexity typically encountered in large-scale analysis of phosphorylation, we developed a novel multi-dimensional fractionation platform that allows for orthogonal separation of phosphorylated peptides in-line with our sensitive electrospray apparatus (unpublished work). This approach provided both sequence identification and relative quantification of over five thousand different phospho-serine/threonine/tyrosine containing peptides from only a few million cells per condition, of which 50 were differentially phosphorylated in the absence of LIF and MEFs (data not shown). We are currently in the process of determining which of these targets are downstream of the ERK1/2 and PI3K/AKT/GSK pathways in mESC, and are actively pursuing the functional significance of these phosphorylation events in driving mESC exit from the pluripotent state.

Collectively our studies with mESC have identified LIF dependent phosphorylation events on a host of proteins in mESC. Though some of these proteins are known to play an essential role in the maintenance of pluripotency, the majority of phosphorylation events we observed downstream of LIF were not known to occur in mESC. Since the initiation of our studies, several groups have reported similar catalogues of phosphoproteins purified from human ESC (hESC) undergoing differentiation¹⁸⁻²¹, and we were pleased to see that several of the most significant pathways that were observed changing in hESC were among those pathways we observed in mESC (data not shown). This suggests that the phosphorylation changes we observed are evolutionarily conserved. Most importantly, the function of many of these conserved phosphorylation events in mESC is entirely unknown in either mESC or hESC. We hypothesize that many of these phosphorylation events are biologically relevant to the transition from a pluripotent mESC to a more restricted germ layer progenitor cell. We are currently determining which of the signaling pathways we observed are functionally important, and are actively pursuing the molecular mechanisms by which they influence mESC differentiation.

Relevance of our work to the development of embryonic stem cell based therapies in the future

Virtually all of the cellular products in clinical use today are derived from the hematopoietic system: red cells, platelets, mature T cells and hematopoietic stem cells. This situation is not coincidental, as their normal physiologic localization and travel via the bloodstream allows for ease of collection (peripheral blood or bone marrow harvest) as well as therapeutic delivery (direct venous infusion). Most importantly, all successful cellular therapies to date are based on stable, relatively long-lived populations of cells that continue their normal development and function once they are infused back in the bloodstream. This is even true for hematopoietic stem cells, which, despite their ability to differentiate into other blood cells, resume their normal and restricted self-renewal functions once they return to their physiologic environment in the adult bone marrow. In a very real sense, the current cellular therapies simply execute their innate physiologic programs in their normal physiologic environment, without any intended direction from our part. We simply move them from one person to another.

In contrast, the hurdles for clinical application of non-hematopoietic, embryonic stem cell populations are much steeper. True embryonic stem cells (ESC) exist for a short time period during embryonic development, and their normal physiologic differentiation occurs within the highly-structured confines of a developing embryo. To date there are no satisfactory methods to fully recapitulate these conditions *in vitro* to generate clinically proven and therapeutically useful populations of tissue-specific cells. In addition to these difficulties, there are many open questions associated with successful engraftment of non-hematopoietic, differentiated cells²². Furthermore, clinical use of tissue-specific cells derived *ex-vivo* from embryonic precursors pose unique health risks. Consider that propagation of ESC requires them to remain in an undifferentiated state for an extended period of time; however, this condition also correlates with their ability to form multi-lineage tumors, or teratomas, when injected into adult animals. As a result there is major concern that tissue-specific cells, derived from stem cell precursors, may be contaminated with highly tumorigenic pluripotent progenitors²³. Thus to fully realize the potential of ESC in cellular therapies, we must first understand the molecular mechanisms that underlie the transition of these cells from a pluripotent to a lineage-restricted state.

The importance of the National Blood Foundation support to Dr. Luckey

The long-term goal of my research has always been to understand what drives cellular differentiation. Historically, I have viewed this problem through the eyes of an Immunologist, and my scientific training focused on understanding CD8⁺ T cell differentiation. This view was altered by my clinical experiences as a Transfusion Medicine physician. I was often confronted by patients with questions about their CD34⁺ hematopoietic stem cells that sounded a lot like those I was asking myself in the lab concerning T cells. How do these cells decide to make more of themselves and self-renew? How do they decide to become a different kind of cell? The more I tried to answer these questions with some authority, the more I realized how little I actually knew. Sure, I knew that external signals were important, but I also realized that ultimately cellular decisions were driven by their transcriptional programs. What I did not know was how external signals became integrated at the epigenetic level; how these signals were “translated” into a given transcriptional program. And so, when I was fortunate enough to be offered an assistant professorship and be given my own lab, I made the decision to not limit myself to the study of T cells, but to also work on understanding these fundamental questions in the arguably the most genetically tractable model for mammalian developmental study, the mouse embryonic stem cell.

Our studies in embryonic stem cells have required us to develop many new tools for their biochemical characterization, including a novel method for their culture on permeable inserts and development of highly sensitive and quantitative mass spectrometry based approaches to phospho-proteomics. The grant I received from the National Blood Foundation played an essential role in supporting our efforts to develop these technologies, and have allowed us to identify multiple targets that link external signaling to the epigenetic control of transcription. The NBF grant further served as the basis for our recent submission of a R01 application to the NIH. Most importantly, the NBF grant provided an external validation of our work and approach at a pivotal time in my career development, providing much needed support to a young investigator venturing into a new area of study.

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Fig 1. Separation of mESC from feeder cells facilitates proteomics analysis

(a) MEFs express more total and phosphorylated (pY705) STAT3 protein compared to mESC. (b) Culture system to transition mESC grown on feeder cells (top) to permeable membranes suspended above MEFs (bottom). Separation of mESC and MEF cultures minimizes contamination in subsequent proteomics analyses and maintains mESC pluripotency as measured by colony morphology (c), Oct4 nuclear localization (d), expression of alkaline phosphatase (e), expression of nanog/Oct4 protein levels of intentionally differentiated mESC (f) vs. mESC grown on inserts above MEFs in the presence of LIF (g), and generation of chimeric mice (h).

Fig 2. Phospho-proteomics workflow

mESC were grown under either self-renewal conditions or in the absence of LIF and MEFs. Resulting peptides were labeled with isobaric (iTRAQ- ABI) tags and mixed. Phospho-tyrosine peptides were enriched by anti-P-tyr antibody immuno-precipitation. Alternatively, phospho-peptides in general were purified by metal affinity chromatography. Resultant peptides were enriched/cleaned-up by reverse phase chromatography. Peptides were introduced to mass spectrometer via a miniaturized liquid chromatography-electrospray assembly. Mass spectrometer was subsequently run in data dependant mode, and MS/MS spectra were used to determine both the sequence and relative abundance of phosphopeptides.

Fig 3. STAT3-Tyr 705 phosphorylation decreases in the absence of LIF/MEFs
 MS/MS spectrum that shows modulation (inset) of STAT3 tyrosine phosphorylation (pY705) in response to LIF/MEF withdrawal. Red and blue dots indicate detection of b- and y-type ions, respectively. (Please note that Fig 3 is reprinted with permission from Reference 14, Ficarro et al *Analytical Chemistry* 2009).