The initiation of mammalian embryonic transcription: to begin at the beginning

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Gamete (sperm and oocyte) genomes are transcriptionally silent until embryonic genome activation (EGA) following fertilization. EGA in humans had been thought to occur around the eight-cell stage, but recent findings suggest that it is triggered in one-cell embryos, by fertilization. Phosphorylation and other post-translational modifications during fertilization may instate transcriptionally favorable chromatin and activate oocyte-derived transcription factors (TFs) to initiate EGA. Expressed genes lay on cancer-associated pathways and their identities predict upregulation by MYC and other cancer-associated TFs. One interpretation of this is that the onset of EGA, and the somatic cell trajectory to cancer, are mechanistically related: cancer initiates epigenetically. We describe how fertilization might be linked to the initiation of EGA and involve distinctive processes recapitulated in cancer.

Measuring mammalian embryonic transcriptional initiation

The first embryonic transcription, EGA (see Glossary), is a critical aspect of the establishment of totipotency [1], yet the initiation and early profile of EGA are poorly understood in any vertebrate species [2]. In fish and amphibian models, the kinetics of EGA are elusively rapid and in mammals, the focus here, embryos have been of indeterminate age, asynchronous, pooled (smoothing expression), or transcript levels obscured by changes in poly(A) tail length [2–5]. Mouse embryonic transcription is thought to initiate by the late one-cell stage (‘minor’ EGA), followed by ‘major’ EGA in two-cell embryos [6]; EGA in humans has been held to begin at the four-to-eight cell stage [7], even though the latency period between fertilization and EGA would have to last at least 2 days. Notwithstanding hints that human EGA initiates earlier than the four-to-eight cell stage [6,8,9], no model accounts for how the embryo genome would be sustained in a transcriptionally silent state, embryonic processes regulated, or the nature of the autonomous cue that triggered transcription.

Resolving these issues would benefit from a detailed profile of transcriptional initiation to enable a model of the underlying mechanism. Accurate determination of transcription immediately after fertilization is confounded by modulation of transcript polyadenylation in early embryos to control translation, which may distort estimates of relative levels [3,5]. This was recently addressed by single-cell RNA-sequencing (scRNA-seq) in human embryos [10]. The method employed a subtractive approach that was independent of transcript polyadenylation status and accommodated the potential for further profile distortion due to maternal RNA inheritance and degradation, in which RNA from the mature, metaphase II (mII) oocyte is transmitted to the embryo during fertilization and subsequently degraded [8,11]. Because scRNA-seq read counts for mII oocytes and embryos required negligible normalization, they were readily comparable, facilitating the detection of EGA [10]. Upregulation was corroborated orthogonally and by conservation with the onset of EGA in mouse one-cell embryos [12]. Moreover, maternal transcript degradation in human embryos is a gradual process occurring over several days following fertilization [8,11], so

Highlights

Our high-resolution single-cell RNA-sequencing revealed that in human embryos, transcription initiates at the one-cell stage, earlier than widely thought.

The new findings suggest that fertilization itself triggers transcription (embryonic genome activation (EGA)) by activating maternal transcription factors.

EGA mostly utilizes canonical promoters and produces normatively spliced, protein-coding transcripts.

The first genes expressed in the embryo predict upregulation by cancer-associated transcription factors, including MYC and MYCN, linking the onset of embryogenesis to cancer.

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its contribution at the one-cell stage is small. Addressing these challenges by scRNA-seq has shown that EGA initiates at the one-cell stage in humans [9]. A discrete set of genes is expressed, suggesting that EGA is programmed, rather than stochastic. We now speculate on the implications of this finding.

**EGA onset predicts the involvement of cancer-associated TFs**

Genes upregulated in EGA [10] can be used bioinformatically to infer the TFs mediating expression. These include the pleiotropic TF, MYC, which binds to many genes, often in cells with regenerative and proliferative potential and affecting metabolic pathways and differentiation [13–15] (see Table 1). MYC is commonly dysregulated in cancer [16], present in mouse mII oocytes [17], and predicted EGA regulators include other cancer-associated TFs, such as MYCN, FOXM1, and E2F4 [10]. MYC often induces small changes in target gene expression, as observed at the onset of EGA [10,12,18]. This low expression amplitude may partly explain why EGA was previously missed in human one-cell embryos and highlights the possibility that cellular potency is commonly modulated by small expression changes [19,20].

Predicted EGA-initiating TFs are regulated by post-translational modifications [21–25]. For example, MYC localization, stability, and activity are sensitive to acetylation [25], isomerization [26], and phosphorylation [24]: cyclin-dependent and MAP kinases phosphorylate MYC [23], whose dephosphorylation is mediated by (among others) protein phosphatase 2A [27]. Several of these are components of phosphorylation pathways regulating Emi2 and Mos to control meiosis in vertebrate oocytes (Figure 1A) [28]. It is also possible that during meiotic progression, de novo translation of maternal transcripts itself contributes to the onset of EGA, as it apparently does for ‘minor’ EGA [29].

Acetylation can directly enhance MYC target gene activation, or do so indirectly, as when MYC recruits histone acetyltransferase (HAT) or deacetylase (HDAC) activities [30]. In addition, MYC can recruit the scaffold protein, TRAPP, to stabilize expression-activating HATs, Tip60 and GCN5 [31], and the cAMP response-element binding coactivator protein, CBP/p300, to modify chromatin and promote MYC acetylation [32,33]. MYC homeostasis also involves the deacetylase, HDAC3 [25]. These and other modifiers and associated HAT and HDAC activities are present in mouse mII oocytes and one-cell embryos [32,34], where they might remodel chromatin after fertilization.

Post-translational modifications of candidate EGA regulators other than MYC include MYCN methylation by protein arginine methyltransferase 1 [35], phosphorylation of the G1-/S-phase mitotic gene expression activator, FOXM1, by polo-like kinase 1 and CDK4/6 [36], and of E2F4 by aurora kinase to regulate DNA binding and subcellular localization [37]. We speculate that together with chromatin remodeling, these modulate TF activity in response to fertilization-induced signaling or chromatin (epigenetic) remodeling, or less directly, as can occur in response to cytoskeletal remodeling or reconfigured phase compartmentalization [38]. In an extension of this model, the onset of embryogenesis is ectopically recapitulated in events that lead to carcinogenesis (Figure 1).

**How are genes marked for the first embryonic transcription?**

At least four mutually inclusive possibilities account for how one-cell embryos designate genes for expression.

First, by association with transcription-activating chromatin. **Topologically associating domains (TADs)** occur in mouse early embryos and could coordinate genome activation by
spatial proximation of EGA genes [39,40]. It is unclear if TADs are preformed in gametes [39] or assemble after fertilization [40], whether the onset of EGA initiates within such domains or, indeed, if early activated genes are clustered and chromatin remodeling seeds expression of nearby genes. Alternatively, activation may occur independently at dispersed loci.

Secondly, via TF cognate DNA sequences. For example, MYC preferentially recognizes the E-box, CACGTG, in a DNA-methylation-sensitive manner [41], whilst E2F4 has the consensus binding site, TTTGCAGC [42]. The default in this second scenario is transcriptional quiescence (the initial state of most genes), with binding of activating TFs to motifs within targeted promoters causing expression of associated genes at the onset of EGA.

Thirdly, meiotic exit triggered by fertilization may result in an epigenetic configuration that is more favorable for transcription at genes upregulated in EGA, likely involving the appearance of histone modifications associated with transcriptional activation [12].

Fourthly, parentally inherited, gamete-borne epigenetic marks might instate transcription. Inherited marks characterize parentally biased, imprinted gene expression in mouse blastocysts.

### Table 1. Functional pathways shared by cancer and the onset of human EGA

| Category                                                                 | Function                          | P value  | z-score | No. | Examples                                                                 |
|-------------------------------------------------------------------------|-----------------------------------|----------|---------|-----|--------------------------------------------------------------------------|
| Cancer, organismal injury, and abnormalities                             | Growth of tumor                   | 7.91e-06 | 4.921   | 105 | EIF2AK3, PRPS1, NAMPT, SP1, GNA13                                        |
| Cancer                                                                  | Cell transformation               | 8.15e-06 | 4.447   | 58  | TEAD1, CCNE1, MAP2K3, GNA13, THRA                                        |
| Cancer                                                                  | Transformation of fibroblast cell lines | 2.01e-07 | 3.678   | 38  | NFKBIA, SMC3, PTTG1, JAK2, AKAP13                                        |
| Cancer, organismal injury and abnormalities                             | Invasive tumor                    | 1.43e-06 | 2.773   | 131 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury and abnormalities                             | Metastasis                        | 1.18e-05 | 2.675   | 109 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury and abnormalities                             | Advanced malignant tumor          | 1.18e-04 | 2.675   | 112 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury and abnormalities                             | Genitourinary carcinoma           | 6.23e-38 | 1.982   | 734 | TIGD5, RGS12, ETVI, LGR4, RPS5K1A                                       |
| Cancer, organismal injury and abnormalities                             | Neoplasia of cells                | 8.51e-23 | 1.818   | 559 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, endocrine system disorders, organismal injury and abnormalities  | Neuroendocrine tumor              | 7.28e-06 | 1.516   | 229 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury and abnormalities                             | Squamous-cell carcinoma           | 5.83e-13 | 1.300   | 370 | TEAD1, CCNE1, BCL6, PTTG1, DICER1                                        |
| Cancer, endocrine system disorders, organismal injury and abnormalities  | Endocrine gland tumor             | 2.17e-39 | 1.152   | 830 | NFKBIA, SMC3, PTTG1, JAK2, AKAP13                                        |
| Cancer, organismal injury and abnormalities                             | Anogenital cancer                 | 1.92e-38 | 1.109   | 733 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury, and abnormalities, reproductive system disease | Mammary tumor                     | 6.15e-22 | 1.030   | 329 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, organismal injury and abnormalities                             | Malignant solid organ tumor       | 4.57e-25 | 1.029   | 701 | RNASSP141, MTC01P12, RIPK4, CDH15, CMTR2                                 |
| Cancer, dermatological diseases and conditions, organismal injury and abnormalities | Skin cancer | 2.50e-24 | 1.029   | 693 | MAB21L1, ETVI, TEB, MAU2, LAMC3                                         |

* Top 15 pathways shared between cancer and upregulated genes in human one-cell embryos as determined by Ingenuity Pathway Analysis (IPA), ranked by z-score (highest uppermost).
* Number of shared genes in the IPA category.
* Top five genes (logFC > 2) in the category.
Figure 1. A model for pathways initiating the onset of embryonic transcription and their recapitulation as a prelude to some cancers. (A) A fertilizing sperm signals via phospholipase C-ζeta (Plcζ) to mobilize available cytoplasmic calcium ions (Ca^{2+}) and trigger a phospho-relay that induces meiotic exit and activates maternally inherited transcription factors (TFs) such as MYC. Remodeling is mediated by meiotic-exit-dependent and -independent pathways and phosphorylation may also directly activate chromatin remodeling (not shown). Some transcription may be potentiated by inherited epigenetic marks (epigenetic inheritance). Expression precedes “major” embryonic genome activation (EGA) two-to-three cell cycles later (upper right). (B) In response to a sufficient trigger, early embryonic conditions of (A) may be recapitulated ectopically, in somatic cells. Subsequent events parallel those at fertilization (where the trigger is provided by a fertilizing sperm), resulting in transient activation of embryonic processes, including gene expression resembling EGA. (Figure legend continued at the bottom of the next page.)
and most correspond to trimethylated histone 3 lysine 27 (H3K27me3) and some to DNA methylation [43]. Parentally inherited marks pass through the one-cell stage, so could influence EGA. Other marks affecting transcription (e.g., H3K4me3) may not correspond to canonical imprints [43,44]. It is also possible that parentally acquired (i.e., nonprogrammed) traits are epigenetically transmitted to offspring to promote gene expression at the one-cell stage. This would exemplify epigenetic inheritance [45] that may not become apparent until adulthood, as can occur in nuclear transfer cloning [46].

Maternal chromatin transitions from a condensed metaphase configuration during meiotic progression, whereas protamines, which comprise 85% of human sperm nucleoprotein, are exhaustively removed and replaced with histones [47]. Differences in parental chromatin thus likely produce distinctive parental gene expression profiles at the onset of EGA.

**Totipotency and the expression trajectory of upregulated transcripts**

A complete model of totipotency will accommodate transcription in one-cell embryos: in humans, transcript levels for most of this set of EGA genes decline with the onset of ‘major’ EGA at the four-to-eight-cell stage [1,7,10]. Although 1~5% of pluripotent mouse embryonic stem (ES) cells are thought to exhibit features of totipotent two-cell embryo blastomeres [48], one-cell embryos are transcriptionally distinct. Transcripts for drivers of gene expression in the cleavage-stage embryo, including pluripotency factors DUX4 and LEUTX, are not upregulated in human one-cell embryos [6,8,10,49]. In addition, TFs upregulated from around the eight-cell stage, including OCT4, SOX2, and NANOG, are not predicted to regulate genes expressed at the one-cell stage [10,50], contrasting with the requirement of pluripotency factors to initiate EGA in zebrafish [51].

Little trace of most transcripts upregulated in human one-cell embryos remains at the eight-cell stage (Figure 1). Maternal transcript degradation, which occurs over several days in human embryos [8,11], may also act on transcripts produced by de novo embryonic expression. However, downregulation of expressed genes is precipitous [10] and apparently coordinated with marked expression of a different set of genes (‘major’ EGA), suggesting that additional degradative mechanisms are responsible. Unlike transcriptional switches, which in many cases are triggered by exogenous cues, as in T cell differentiation, neurogenesis, and pre-implantation lineage specification, the programmed switch to ‘major’ EGA is autonomous at the level of the embryo.

Genes expressed in one-cell embryos may encode essential early developmental pathways. Inhibiting RNA polymerase II (Pol II) in mouse one-cell embryos acutely blocks development and pathway analysis of expression in human one-cell embryos maps upregulated genes to key processes, such as kinetochore, ATM, and GADD45 signaling, DNA damage checkpoint regulation, cytokinesis, metabolism, and cancer pathways [10]. Some of these reflect conserved and ubiquitous cellular characteristics and it may be no surprise that they are upregulated in a newly formed cell type upstream of proliferation. But the processes may also involve pathways peculiar to early embryos (several genes await annotation, indicative of embryo specificity) that are expressed ectopically to engender cancer much later. Another speculative paradigm is

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These events may be abortive, reverting the cell to its original phenotype (or maintaining the original phenotype), or lead to disruption of key homeostatic pathways that predispose the cell to metabolic flux and genomic instability, followed by selection, progression, and founding of the primary cancer (P0), possibly as a multipotent stem cell. The initiating event is epigenetic and transient: there is no trace of the profile that resembled the onset of EGA by the time cancer is detectable. The period between initiating events and the cell fate commitment that produces a P0 primary founder cell (upper right) may be brief given that it is denoted by transient chromatin modification.
regulation of the Warburg effect in cancer cells [52,53]. Although genes for glycolytic enzymes that mediate the Warburg effect (e.g., enolase 1 and triose phosphate isomerase) are not upregulated in one-cell embryos, they are soon afterwards, in cleavage-stage development. One-cell embryos may thus anticipate the metabolic transition that presages cancer.

In addition to early embryonic roles, one-cell stage expression may be required subsequently for healthy development into adulthood, reflecting a delayed impact. Precedents in which otherwise apparently healthy preimplantation embryos produce adult-onset phenotypes include (in the mouse) disruption of the imprinted gene, Zdbf2 [54], and nuclear transfer cloning [46]. Early expression could subtly impact later transcription or other processes: downstream consequences of gene expression at the one-cell stage, but which are not evident in blastocysts or ES cells, would impact models of gastrulation and postimplantation development. This is relevant to the debate about the ‘14-day rule’ relating to in vitro culture of human embryos beyond 14 days [55], in that it necessitates demonstration that the onset of EGA had occurred sufficient to support healthy development.

Normative transcript expression and splicing in human one-cell embryos

Notwithstanding a report that mouse EGA is promiscuous and uncoupled from splicing [56], new transcripts in human one-cell embryos reflected canonical Pol II start sites; they were canonically spliced to produce potentially functional transcripts and intron-flanking qPCR corroborated splicing of upregulated transcripts. Most were protein-coding and transcription reflected early embryonic pathways [10]. This is consistent with findings in the mouse [12].

Relevant to splicing, one-cell embryos lack nuclear membranes for several hours following fertilization: maternal chromosomes persist in a nonenveloped state and sperm are stripped of plasma membrane and intrinsic nuclear envelope [33,57,58]. Spliced transcript formation during this ‘pre-pronuclear’ phase awaits confirmation, but nuclear pore complexes (NPCs) appear early on chromatin after gamete fusion in cattle and, in the mouse, the NPC assembly factor, ELYS, localizes to peripheral chromatin during paternal pronuclear formation [59]. Several NPC components have been identified in mouse one-cell embryos, including nucleoporin, and NPC assembly is disrupted in developmentally arrested human one-cell embryos [60,61]. The picture is that once formed, pronuclei contain NPCs that could support pre-mRNA splicing during transcript export.

A model for transcriptional initiation in one-cell embryos

A working model of EGA in mammalian one-cell embryos will integrate transcriptional onset with the events of oocyte activation in fertilization [47]. Sperm–oocyte union rapidly triggers an increase in the available cytoplasmic Ca^{2+} ion concentration ([Ca^{2+}]) that, in the mouse, oscillates over several hours [62]. Fluctuating [Ca^{2+}], modulates TF activity in multiple intracellular contexts [63–65]. For example, gene expression in cardiomyocytes is regulated by Ca^{2+}-sensitive calmodulin kinase II (CaMKII) [63], and CaMKII\text{y} is thought to promote meiotic exit in mammals by phosphorylating Emi2 [28,66]. The phospho-relay that triggers meiotic progression could activate MYC, MYCN, FOXM1, E2F4 [21–24], and other maternal TFs in response to fertilization. This is illustrated by FOXM1-mediated trans-activation, which requires binding of CDK-cyclin complexes for phosphorylation-dependent recruitment of coactivator, p300/CBP [67]. Conversely, phosphorylation primes MYC by ERK and CDK1 for proteosomal degradation [23] and might act as an ‘off’ switch that parallels Emi2 degradation after fertilization [28,66]. As well as canonically spliced protein coding mRNA transcribed by Pol II, the model (Figure 1A) accommodates the possibility that other RNA polymerases and noncoding RNAs are analogously regulated in one-cell embryos.
In experimental settings, meiotic exit can support full and efficient development without Ca\(^{2+}\) mobilization [68], so Ca\(^{2+}\) mobilization is not a *sine qua non* to initiate essential transcription in EGA. It is unknown how EGA in the absence of Ca\(^{2+}\) release relates to EGA in physiological fertilization: presumably, Ca\(^{2+}\)-independent aspects of meiotic exit are able at least to initiate a minimal EGA sufficient for full-term development. It is also notable that parthenogenetic mouse embryos efficiently support development when sperm are injected during the first mitotic metaphase, with dysregulation of a minority of genes [73] by the two-cell stage [58]. One interpretation of this is that the onset of EGA primarily involves expression from the maternal genome [12], but it also suggests plasticity in the relationship between fertilization and the onset of EGA, with relevance to parallels in cancer (Figure 1B).

Post-translational control of MYC and other regulatory TFs may modulate gene expression in one-cell embryos. Some of the processes involved are likely to be anomalous: MYC itself may serve to illustrate this, because whilst it typically has a half-life of \(~20\) min [69], as a maternal factor mediating EGA it is likely to be more stable in mII oocytes and early embryos [12]. Although paternal chromatin assembly and genomic DNA demethylation do not require meiotic exit overall, some remodeling does [34] and we suggest that fertilization triggers post-translational modification to activate reprogramming and remodeling complexes. Assigning roles for remodeling factors in one-cell embryos is confounded by maternal inheritance: genetic deletion may induce compensation [70] or not become phenotypically manifest until the maternal supply of corresponding transcript or protein is exhausted, masking essential roles in one-cell embryos. From information available in the mouse, SWI/SNF remodelers are expressed in preimplantation embryos [71] and the ISWI ATPase Snf2h is required for preimplantation development [72], so these and other remodeling activities may play formative roles in the instatement of EGA.

**Concluding remarks**

A model of EGA initiation emerges from recent work in which cues during fertilization activate maternal TFs such as MYC (Figure 1). Activated TFs initiate an embryonic program of transcription by Pol II, enabled by permissive chromatin at expressed gene promoters, either because the chromatin was already present in gametes, or due to modification in response to meiotic exit, by remodeling factors from the oocyte. Evaluation of these possibilities awaits the development of sufficiently robust and sensitive protocols for consensus chromatin analysis at single-cell scale. Genes expressed at the onset of EGA encode pathways essential for genome integrity and other key early embryonic processes. We suggest that these can be reactivated ectopically in somatic cells much later, as a Prelude to cancer. This pertains to cancer that does not initiate from genetic mutation, as in heritable cancers, but rather, is essentially epigenetic. In the model, the initiating event, for example, a traumatic cellular insult, engenders a state with key features resembling that of a one-cell embryo, including a gene expression program reminiscent of EGA initiation, resulting in the disruption of cytokinesis [73], metabolism [52], and DNA homeostasis [74,75]. These potentiate transformation in somatic cell division, energy usage, and genome stability, leading to cancer. Abrogating genome integrity would promote cumulative mutations in the founder cancer cell, subsequently producing a mutational signature. TFs shared between the onset of cancer and embryogenesis promise mutually to reveal critical components of each (Figure 1B). Future work will seek to understand the etiological connection between the predicted state resembling embryonic totipotency in somatic cells and the earliest cancer states in clinical samples (see Outstanding questions). A major challenge will be to identify ‘quasi-totipotent’ somatic cells predicted by the model (Figure 1), since even if they occur and represent cancer precursors, they would no longer be extant by the time cancer is manifest.
Declaration of interests
No interests are declared.

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