Generation of embryos directly from embryonic stem cells by tetraploid embryo complementation reveals a role for GATA factors in organogenesis

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Abstract
Gene targeting in ES (embryonic stem) cells has been used extensively to study the role of proteins during embryonic development. In the traditional procedure, this requires the generation of chimaeric mice by introducing ES cells into blastocysts and allowing them to develop to term. Once chimaeric mice are produced, they are bred into a recipient mouse strain to establish germline transmission of the allele of interest. Although this approach has been used very successfully, the breeding cycles involved are time consuming. In addition, genes that are essential for organogenesis often have roles in the formation of extra-embryonic tissues that are essential for early stages of post-implantation development. For example, mice lacking the GATA transcription factors, GATA4 or GATA6, arrest during gastrulation due to an essential role for these factors in differentiation of extra-embryonic endoderm. This lethality has frustrated the study of these factors during the development of organs such as the liver and heart. Extraembryonic defects can, however, be circumvented by generating clonal mouse embryos directly from ES cells by tetraploid complementation. Here, we describe the usefulness and efficacy of this approach using GATA factors as an example.

Embryos can be generated directly from ES (embryonic stem) cells

The availability of mouse ES cells and the finding that genes could be efficiently manipulated through homologous recombination in these cells has facilitated the use of reverse genetics in mice to reveal mammalian gene function. The relative ease of the procedure has led to an explosion in the generation of mouse strains harbouring genetic alterations. Usually the primary function in the mouse of any given gene is initially addressed by attempting to generate a loss-of-function allele, although more subtle allelic variations such as mutation of phosphorylation sites and transcriptional-regulatory elements are becoming more common. A major theme in biology, however, has revealed that proteins with crucial regulatory roles are commonly utilized in many seemingly diverse biological pathways. For example, FGF (fibroblast growth factor) signalling has important roles in development of the liver, heart and limbs and in addition has physiological importance [1–4]. Gene knockout studies, however, only provide information regarding the first essential role of a protein. Being limited to addressing the primary role of a protein can be problematic, especially if one is studying a protein’s contribution to organ development. This is especially true of proteins that are expressed in extra-embryonic tissues.

During gastrulation, when the primary tissue types of the foetus are formed, the role of the extra-embryonic endoderm is crucial. If formation of the extra-embryonic endoderm is compromised, then gastrulation arrests and subsequent development of the foetus is perturbed [5]. The extra-embryonic endoderm shares functions with the definitive endoderm, a tissue that ultimately gives rise to organs such as the gastrointestinal tract, liver, pancreas and lungs. Many of the genes that have important roles in development of these endoderm-derived organs also act to control differentiation of the extra-embryonic endoderm. This means that analyses of genes that have potential roles in the development of endodermal organs are often exacerbated by the finding that these same genes are necessary for extra-embryonic endoderm function. In addition to genes encoding proteins with roles in controlling extra-embryonic endoderm development, the use of gene knockouts is insufficient to study genes in which a haploinsufficiency results in embryonic lethality. For example, deletion of one allele encoding VEGF (vascular endothelial growth factor) disrupts vascular development in the placenta causing a developmental arrest [6]. Lethality associated with haploinsufficiency means that a founder mouse that is heterozygous for the disrupted allele cannot be established, thereby preventing analyses of the heterozygous phenotype.

Some of the limitations associated with generating ubiquitous gene knockouts have been circumvented by the use of the Cre/loxP system. In this system, loxP elements are inserted into the genome, through homologous recombination in...
ES cells, such that they flank a genomic sequence that is destined to be deleted. Animals that are homozygous for such a floxed (flanked by loxP) allele are usually physiologically normal, because the loxP sites are positioned within non-coding regions of the genome. However, the enzyme Cre recombinase has the capacity to mediate recombination between tandem loxP elements, resulting in the deletion of intervening DNA. This means that if a transgene expressing Cre from a cell-type specific promoter is introduced into homozygous floxed animal, then the target gene will be deleted in a cell-type-specific manner. While this technique has been enormously powerful to look at tissue-specific gene function, it also has a number of disadvantages. Foremost, successful deletion of a target gene requires the availability of strains of mice in which Cre is expressed in a suitable profile. Although the number of available Cre strains is increasing, this is still a significant limitation to the system. The second limitation is that the process is extremely time consuming because multiple breeding cycles are required to generate an experimental animal. These limitations mean that there is need for efficient procedures that can circumvent lethality associated with loss of extra-embryonic endoderm function or haploinsufficiency, and facilitate the generation of mice and embryos harbouring genetic modifications.

One suitable procedure that is simple and efficacious under such circumstances is the generation of mice and embryos directly from ES cells by tetraploid embryo complementation [7–9]. In this procedure, illustrated in Figure 1, the blastomeres of two-cell embryos are fused to generate one-cell tetraploid embryos. These embryos can be cultured to morula stages of development and then combined with diploid ES cells either by aggregation or by direct injection of ES cells under the zona pellucida. The resulting chimaeric preimplantation embryos can develop to term after transfer to a surrogate mother. Lineage tracing experiments have shown that during early stages of development, the cell lineages derived from the tetraploid embryos and diploid ES cells diverge such that the foetus or offspring is derived solely from the ES cells while the extra-embryonic endoderm is generated from the tetraploid cells (Figures 1B and 1C) [8]. In essence, this approach can be used to generate populations of genetically identical mice directly from ES cells. If the ES cells are homozygous for any genetic alteration, then phenotypes associated with the allele will manifest in the ES cell-derived offspring. Moreover, because the extra-embryonic endoderm is derived from the tetraploid embryo, using wild-type embryos as tetraploid donors facilitates the rescue of gene defects that cause extra-embryonic endoderm dysfunction. While this procedure seems like the ideal method to generate mice with genetic alterations, the efficiency with which ES cell-derived offspring could be recovered was originally relatively low (<10%). However, recent work has established that the efficiency of obtaining liveborn offspring from ES cells by tetraploid embryo complementation increases significantly when the ES cells are from an outbred mouse strain, presumably as a consequence of hybrid vigour [10].

Figure 1 | Defects in extra-embryonic endoderm differentiation can be rescued by tetraploid embryo complementation

(A) A schematic representation showing the generation of tetraploid embryos by electrofusion of two-cell embryos isolated from ROSA26 mice that ubiquitously express LacZ. These were aggregated with wild-type diploid ES cells and allowed to develop in utero until E 9.5, at which point foetuses were stained for β-Gal expression. (B) Tetraploid LacZ-positive-derived tissue is found only in the extra-embryonic endoderm, while the foetus is derived solely from ES cells. (C) Section of ES-derived embryos showing LacZ in extra-embryonic endoderm (arrow).

Transcription factors GATA4 and GATA6 have essential roles in extra-embryonic endoderm function

Several lines of biochemical and molecular evidences have implicated the zinc finger transcription factors GATA4 and GATA6 in controlling embryonic development of the heart, gastrointestinal tract and liver [11–14]. Unfortunately, a direct role for these factors in organogenesis has been difficult to address in mammals, because gene knockouts of either GATA4 or GATA6 cause early embryonic lethality [15–18]. Analyses of the expression profile of these GATA factors revealed that both were expressed in the extra-embryonic
endoderm suggesting that the early developmental arrest associated with the loss of GATA4 or GATA6 could be due to deficiencies in extra-embryonic endoderm function [15,16,18,19]. If this were true, it would imply that embryos lacking either GATA4 or GATA6 should complete gastrulation, if these were embryos that provided a wild-type extra-embryonic endoderm by tetraploid embryo complementation. To address this, ES cells were produced that were homozygous for null alleles of either GATA4 or GATA6 using conventional gene-targeting procedures [20–22]. These ES cells were then aggregated with wild-type tetraploid embryos and allowed to develop to in utero. In contrast with knockout embryos, both Gata4−/− and Gata6−/− ES cell-derived embryos completed gastrulation and developed until around E 10.5 (embryonic day 10.5) [20–22]. Genotyping of these embryos confirmed that they lacked a wild-type Gata4 or Gata6 gene respectively [20,21]. These data irrefutably confirmed that both GATA4 and GATA6 are required in the extra-embryonic endoderm to generate an environment that supports gastrulation.

Analyses of Gata4−/− and Gata6−/− ES cell-derived embryos reveal a requirement for these factors in the development of multiple organs

The ability to rescue GATA4 and GATA6 null embryos from early embryonic lethality afforded the opportunity to study the role of these factors during embryogenesis. We initially focused on hepatogenesis, because molecular studies had demonstrated that the GATA factors may have important roles in regulating liver gene expression during early embryogenesis [23–25]. At E 8.5, when the endoderm is being induced to adopt a liver cell fate, hepatic markers were detected in isolated Gata4−/− or Gata6−/− ventral endoderm using RT (reverse transcriptase)–PCR [20,21]. However, slightly later in development at E 9.5, it was found that hepatic development was arrested in both GATA4 and GATA6 null embryos, which were unable to generate a normal liver bud [21,26]. This block to liver development was more severe in GATA6 null embryos, which at E 9.5 showed no indication of expression of albumin mRNA or HNF4α (hepatocyte nuclear factor 4α) protein, both of which are early markers of commitment of the endoderm to a hepatic fate. From these studies we concluded that GATA6 is dispensable for the onset of hepatogenesis, but is essential for the endoderm to commit to a hepatic developmental programme [26]. In GATA4 null embryos, the liver bud also failed to expand; however, in contrast with embryos lacking GATA6, both albumin and HNF4α could still be detected within the rudimentary hepatic primordium [21]. In addition to defects in hepatogenesis, we found that development of the heart, procordial and septum transversum mesenchyme was also disrupted in Gata4−/− embryos [21]. The disruption to the development of septum transversum mesenchyme is particularly interesting, given the proposal that the septum transversum is a source of secreted signals that control early liver bud formation [27]. The use of ES cell-derived embryos has therefore given the first insight into the crucial contribution of GATA factors in controlling multiple aspects of organ development in the mouse.

References
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