Gene expression in the mouse preimplantation embryo

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Mouse preimplantation development represents a tightly controlled programme of gene expression and cell division, which starts with the fertilized egg and ends with implantation of the blastocyst approximately 4.5 days later. Spatial and temporal differences in gene expression underpin establishment of axes at the two-cell stage and development of the trophectoderm and inner cell mass after embryo compaction at the eight-cell stage. Approximately 15 700 mouse genes expressed during preimplantation development have been identified from cDNA sequences deposited in the UniGene database of the National Institutes of Health. This inventory of preimplantation genes is the starting point for identifying signalling modules that function in preimplantation development.

Important patterning events occur during mouse preimplantation development, which starts with the fertilized egg and ends with implantation of the blastocyst approximately 4.5 days later. Compaction at the eight-cell stage followed by asymmetric cell division creates an outer epithelial cell layer that surrounds the inner cell mass (ICM) and allows formation of the blastocoel. Superimposed on these large-scale changes is the development of axes that anticipate the body axes of the implanted embryo.

There are a number of experimental features that make mouse preimplantation development an attractive model for studying mammalian differentiation and patterning: (i) the ability to culture fertilized eggs through to blastocysts *in vitro*; (ii) embryonic staging by visual inspection; (iii) development in simple media without extrinsic cues; and (iv) a large catalogue of expressed genes.

This review is divided into three sections. The first deals with the use of computational genomics to produce an inventory of about 15 700 mouse preimplantation genes. The second section outlines briefly the current status of preimplantation development, and the third section discusses the potential of functional genomics to identify key genes that play a role in this development.

Establishing a draft inventory of genes expressed in mouse preimplantation development

Mammals possess about 200 different types of cell. Large numbers of expressed sequence tags (ESTs) have been obtained for many of these types of cell through the construction of complementary DNA

(cDNA) libraries derived from tissues and organs. Most of these ESTs have been derived from either mouse or human material. Repositories of ESTs include the dbEST database at the National Institutes of Health (NIH; <http://www.ncbi.nlm.nih.gov/dbEST/>) and the RIKEN database (<http://genome.gsc.riken.go.jp>). This review focuses on the use of ESTs imported into the UniGene database (Boguski and Schuler, 1995; Schuler, 1997). This is for two reasons. First, relatively few RIKEN transcript sequences are derived from cDNA libraries for early embryos in mice (see the RIKEN mouse cDNA encyclopaedia at <http://genome.gsc.riken.go.jp>) and, second, most RIKEN transcript sequences in the public domain have been incorporated into the UniGene database, making the UniGene database the more comprehensive of the two.

The NIH UniGene database contains ESTs and mRNA sequences imported from the dbEST database. UniGene clusters these ESTs by searching for sequences shared in common. In principle, matching sequences will be present between transcripts from the same gene but not between transcripts from different genes. (For a fuller explanation of the methodology, see <http://www.ncbi.nlm.nih.gov/UniGene>). If properly constructed, the number of UniGene clusters should approximate to the number of genes in the genome. UniGene clusters are built from ESTs and mRNAs irrespective of their tissue source and large UniGene clusters may contain ESTs contributed by many tissues. At the present time, the human UniGene database (build no. 157) contains about 4×10^6 ESTs and mRNAs, which cluster to 115 523 UniGenes. Figures for a recent mouse UniGene build (no. 118) are about 2.6×10^6 ESTs and mRNAs, generating 87 543 clusters.

The overall number of genes in both the human and mouse genomes is a matter of considerable interest

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and one that is relevant to this discussion. The number of human genes has been estimated to be between 30 000 and 81 000 (Ewing and Green, 2000; Liang *et al.*, 2000; Roest Crolius *et al.*, 2000; Lander *et al.*, 2001; Venter *et al.*, 2001; Wright *et al.*, 2001; Zhuo *et al.*, 2001). (The much-quoted figure of 120 000 genes in the title of the paper by Liang *et al.* (2000) was subsequently corrected to 81 273 genes (see Corrections in *Nature Genetics* **26** 501 (2000)). By contrast, a recent analysis of mouse cDNAs produces a minimum estimate of 70 000 transcriptional units for the mouse genome (Okazaki *et al.*, 2002). (Transcriptional units are clusters of transcripts that contain a common core of genetic information. They include alternatively spliced transcripts but may not always be protein-coding RNAs.) There are several reasons for differences between the number of genes estimated by computational methods and by expressed sequence methods. One difference stems from biases in the training sets used for optimizing gene-finding algorithms which limit the types of genes that are predicted computationally. A second difference stems from the distinction between genes for protein-coding sequences and genes for other types of transcript. Given the rigour of several recent EST clustering analyses including, in some cases, mapping of putative clusters to the genome, the higher figures for the number of genes (as transcriptional units) appear to be robust. There is now reasonable consistency in the number of genes estimated from expressed sequence (65 000–81 000 genes for humans (Liang *et al.*, 2000; Wright *et al.*, 2001; Zhuo *et al.*, 2001) and 70 000 for mice (Okazaki *et al.*, 2002)).

One of the key findings from analysis of gene expression in mice is the presence of significant amounts of non-protein-coding RNAs, in addition to protein-coding, alternatively spliced, polyadenylated, and initiated RNAs, sense and antisense RNAs and RNA-edited transcripts (Okazaki *et al.*, 2002). Most of these transcripts will be captured by conventional methods of capturing RNA and will contribute to any computational system for clustering transcripts, such as UniGene. The current number of UniGenes for the mouse (87 543) is broadly commensurate with the number of genes recently estimated for the mouse using analysis of cDNAs (Okazaki *et al.*, 2002), but the number of human UniGenes (115 523) is considerably larger than corresponding estimates. There may be a number of reasons for this disparity, including the possibility that human UniGene clusters that contain only one EST ('singletons') represent rare transcripts from genes with very low expression.

Two key features of UniGene databases that are fundamental to the analysis of gene expression in mouse preimplantation development are discussed in this review. First, many of the ESTs contributing to UniGene are derived from cDNA libraries that individually contribute large blocks of ESTs from defined tissue sources. Second,

each originating cDNA library retains a dbEST library catalogue number and this number is preserved in each UniGene EST entry, making it possible to track and amalgamate distinct bodies of expressed sequence data from identifiable tissue sources (Stanton and Green, 2001a).

The UniGene databases for mice and humans are now very large and require specialized software to handle them if maximum value is to be extracted. A suite of programmes has recently been developed using Bioperl software, which allows rapid automated downloading and handling of UniGene libraries and integration with downloads of the entire UniGene and HomoloGene databases (J. L. Stanton, A. B. Macgregor and D. P. L. Green, unpublished). (These programs replace earlier library derivations using FileMaker Pro (Stanton and Green, 2001a,b, 2002). Note also that Stanton and Green (2001a,b, 2002) referred to UniGene library IDs. These UniGene IDs have now been replaced in the databases by dbEST library IDs. The conversion table is available from <ftp://ftp.ncbi.nih.gov/repository/UniGene/> (file name Mm.UGLID_dbestLID)).

The mouse UniGene database contains about 150 dbEST libraries that are identifiably embryonic in origin and eighteen of these libraries are derived from stages of preimplantation development (unfertilized egg, fertilized egg, two-cell, four-cell, eight-cell and 16-cell embryos, and blastocyst). Collectively, these 18 libraries contain about 102 000 ESTs and mRNAs and generate about 15 700 UniGene clusters. Subject to the limitations of UniGene, this represents a draft inventory of mouse preimplantation genes. Not all these UniGenes will be for protein-coding RNAs.

The clear staging of the contributing cDNA libraries (for example, egg, two-cell embryo) allows use of these libraries to identify major changes in gene expression across the period of preimplantation development (Ko *et al.*, 2000; Stanton and Green, 2001a,b, 2002). The absence of contamination by other cells also allows assignment of the entire gene expression profiles to defined types of cell; a single type in the case of the egg and two types of cell in the case of the blastocyst. This has enormous advantages in analysis of gene expression and places these particular dbEST libraries apart from most others in the UniGene database, which are derived from complex mixtures of types of cell.

Data derived from the eighteen preimplantation libraries can be used in two ways. First, they can be analysed for the UniGenes they contain to produce 'parts lists' (Stanton and Green, 2001b, 2002). For example, libraries can be amalgamated to generate inventories for individual preimplantation stages (for example, unfertilized egg, fertilized egg) or amalgamated to create a single composite inventory of genes expressed in preimplantation development (Stanton and Green, 2001b, 2002). Such inventories are becoming increasingly important as a starting point for identifying, for

example, signalling pathways and gene expression circuitry. A number of these genes have orthologues in other species (notably humans, but also rats and rabbits) and these can be identified by mapping the preimplantation inventory of mice onto HomoloGene (<http://www.ncbi.nlm.nih.gov/HomoloGene/>; Stanton and Green, 2001b, 2002), which provides a robust inventory of orthologous genes that has the benefit of the extensive annotation of function available for many human genes. However, this comparative exercise also underscores the absence of many of the human orthologues of mouse embryonic genes in the human expressed sequence databases. A major future use for mouse embryonic sequence data is therefore to act as electronic probes of human and other mammalian genomes to identify as yet undiscovered orthologous developmental genes.

The second use of the eighteen mouse preimplantation libraries is to generate profiles of gene expression for the stages of preimplantation development and to use these profiles to detect differences in gene expression that are statistically significant (Stanton and Green, 2001a). Again, this comparative approach, which was originally performed with FileMaker Pro, is now performed rapidly and automatically using Bioperl software (J. L. Stanton, A. B. Macgregor and D. P. L. Green, unpublished). This use of UniGene databases has the great advantage that it gives direct access to changes in gene expression without the need to wait for DNA array or quantitative PCR data.

To summarize this section, the computational approaches discussed above provide a large draft inventory ('parts list') of mouse preimplantation genes. Information about these genes ranges from fragments of cDNA sequence without identifiable reading frames to full-length transcripts with well-defined protein products and substantial additional annotation. A significant number of annotated preimplantation genes have already been identified (Green and Stanton, 2001b, 2002) using mapping of mouse inventories onto HomoloGene, although most of these genes are well-established genes used by a range of types of cell. What is lacking, at present, is full-length sequence data for many of the genes for which expression occurs predominantly in embryogenesis and particularly in the preimplantation embryo.

Despite this deficiency, the gene inventory is large enough to support a substantial functional genomics approach to mouse preimplantation development. The key developmental events that are underpinned by expression of this inventory of genes is briefly outlined below, before a discussion of some possible directions for this approach.

Morphology of mouse preimplantation development

Two key questions in mammalian preimplantation development are (i) what accounts for the emergence

and developmental fates of the trophectoderm and ICM; and (ii) what accounts for the development of axes in the preimplantation embryo that anticipate the axes of the postimplantation embryo? To address these questions in molecular terms requires an understanding of the morphology of preimplantation development.

The unfertilized mouse egg is spherical, with an animal-vegetal (A-V) axis in which the animal pole is marked by attachment of the second polar body (Fig. 1a). This axis remains identifiable in the blastocyst (Fig. 1d,e). Mouse eggs are polarized along the A-V axis; the animal pole lies at the centre of a cap of plasma membrane, which is microvillus-free whereas the remainder of the egg is covered in microvilli.

Recent evidence indicates that axes that anticipate those of the embryo proper become specified at first cleavage or earlier (Weber *et al.*, 1999; Gardner, 2001a; Zernicka-Goetz, 2002). First cleavage occurs in a plane that approximately bisects the fertilized egg and passes close to or through the A-V axis (Gulyas, 1975; Howlett and Bolton, 1985; Gardner, 2001b; Piotrowska and Zernicka-Goetz, 2001). This plane also defines an orthogonal axis that passes through the two half blastomeres (Fig. 1b). When the blastocyst is formed after several further cell divisions, its embryonic and abembryonic poles lie along this same orthogonal axis (Ciemerych *et al.*, 2000), with the site of the first cleavage plane marking the boundary between polar (embryonic) and mural (abembryonic) trophectoderm (Fig. 1d). Evidence indicates that the first two-cell blastomere to undergo second cleavage forms the embryonic pole, and the second blastomere forms the abembryonic pole (Piotrowska *et al.*, 2001). The polarity of the A-V axis is also preserved in the blastocyst. A cross-section of the blastocyst in the plane separating embryonic and abembryonic poles is elliptical (Fig. 1e), with the major axis (the original A-V axis) marked by the second polar body (Gardner, 1997).

It is clear from these observations that the first cleavage division produces dissimilar half blastomeres. How this dissimilarity arises is still a matter of conjecture. The ovulated egg may contain an asymmetric distribution of specific mRNAs or transcription factors which results in differences at the two-cell stage. Alternatively, uneven allocation of components such as the cytoskeleton may cause the asymmetry. As mentioned previously, second cleavage is asynchronous (Gulyas, 1975; Piotrowska *et al.*, 2001), with the first half blastomere to divide contributing predominantly to the embryonic part of the blastocyst (Piotrowska *et al.*, 2001).

The other important conclusion to be drawn from these observations is that the site of blastocoel formation is an intrinsic property of the polarity established at the two-cell stage. The specification of the position of the cavity must therefore be due to cellular events that are set in train by first cleavage. This is a subtle effect imposed on the much more obvious determination of

Fig. 1. Diagrammatic representation of four stages of mouse preimplantation development. The site of the second polar body (grey) and animal-vegetal (A-V) axis are shown in the same orientation throughout. (a) Fertilized egg showing both pronuclei. The future embryonic-abembryonic (Em-Ab) axis lies orthogonal to the A-V axis. (b) Two-cell embryo showing A-V axis and future Em-Ab axis. (c) Compacted 16-cell embryo. (d) Blastocyst shown in cross-section in the plane of the A-V and Em-Ab axes, with the second polar body marking the boundary between polar and mural trophoblast. (e) Cross-section of the same blastocyst rotated at right angles to (d), showing the ellipticity of the blastocyst along the A-V axis.

cell fate shown by the trophoblast and ICM. Other evidence indicates that the first cleavage plane passes close to or through the site of sperm fusion (Piotrowska and Zernicka-Goetz, 2001; Plusa *et al.*, 2002). Mouse spermatozoa can apparently fuse anywhere on the 80% of the egg surface covered in microvilli.

The pattern of cell division at second- and third-cleavage divisions needs careful re-examination in the light of these data. The first of the two cell divisions at second cleavage occurs meridionally, in a plane that aligns approximately with the former A-V axis of the egg (Fig. 2). The second of the two-cell divisions starts in the same plane but this plane turns relative to the first two-cell division as cytokinesis proceeds, resulting in a plane separating the second pair of quarter blastomeres that lies at right angles to the first. This positions the four quarter blastomeres as a tetrahedron. Interestingly, the rotation of the second plane with respect to the first tends to occur preferentially in one direction in the rabbit, the only species in which it has been studied (Gulyas, 1975). This introduces chirality, with each of the quarter blastomeres potentially having different developmental potential. It is unknown whether

this biased rotation occurs in mice and humans, and whether it has developmental significance.

The four-cell embryo cleaves to give the eight-cell embryo, which undergoes compaction and formation of tight junctions shortly after third cleavage (Ziomek and Johnson, 1980; Johnson and Ziomek, 1981). Compaction produces apical and baso-lateral surfaces. Although each eight-cell blastomere has the appearance of equivalence, they cannot be equivalent in the undisturbed embryo as the eight-cell embryo carries an incipient embryonic-abembryonic (Em-Ab) axis (Ciemerych *et al.*, 2000). Cell division of the eight-cell embryo is geometrically heterogeneous, with some cells undergoing lateral division and some dividing along a radial axis. On average, about twice as many cells undergo lateral cell division as radial division, giving about a 2:1 ratio of outer to inner cells at the 16-cell stage (Balakier and Pedersen, 1982; Fleming, 1987). Blastocoel formation begins at 32 cells and occurs in the abembryonic half of the Em-Ab axis established at first cleavage.

Late in blastocyst development, the ICM differentiates into epiblast and primitive endoderm. After implantation,

the polar trophoctoderm expands towards the abembryonic pole to form the extra-embryonic ectoderm and the primitive endoderm expands to become visceral and parietal endoderm, with epiblast lying between the two (see Beddington and Robertson, 1999). Endoderm originating from ICM cells close to the second polar body contributes to the proximal end of the egg cylinder whereas endoderm derived from cells at the far end of the major axis contributes to the distal end (Weber *et al.*, 1999). The proximodistal axis of the egg cylinder descends directly from the Em–Ab axis of the blastocyst (Weber *et al.*, 1999).

Gene silencing: screening for preimplantation phenotypes

The large inventory of mouse preimplantation genes described has the potential to be a major tool in understanding preimplantation developmental processes. One way in which it can be of value is to identify candidates for gene silencing, as, by silencing key genes, aberrant phenotypes that indicate functionally important mechanisms may be identified. This section begins with a discussion of the results of gene silencing using conventional knockout technology.

Numerous laboratories have produced mouse homozygous null mutations over the past 15 years that show lethal effects on mouse development; those that show a pre- or peri-implantation phenotype, principally lethality, are listed (Table 1). The appearance of a phenotype in homozygous null mutant embryos produced by mating heterozygous $\pm$ carriers depends on eliminating maternal transcripts and then eliminating the translated protein. The earliest sign of any effect is shown by *Rad51* and *profilin 1* null mutants, which are lethal by first cleavage (Tsuzuki *et al.*, 1996; Witke *et al.*, 2001). This indicates that rapid turnover of maternal mRNA and protein after fertilization is possible. However, a significant number of null mutants show no lethality until embryonic day 4 (E4.0) or beyond, indicating either a much slower elimination of maternal transcripts and protein or a later onset of normal expression for these genes. In some cases, a mutant initially produces a hypomorphic phenotype which is then followed by embryonic death some days later (for example, *Blm*; Chester *et al.*, 1998).

These data indicate that a pre- or peri-implantation phenotype can be caused by targeted gene silencing. All of the genes that have been reported as producing preimplantation lethality when silenced are found in the inventory of about 15 700 preimplantation genes. Moreover, proteins that are physically associated with the products of these genes also appear in the inventory. Some examples are discussed here.

One of the more striking examples is shown by the four separate genes (*Rad50*, *Brca1*, *Nbn* and *Blm*), all of which cause early embryonic lethality when deleted.

Fig. 2. Diagrammatic representation of the first two cleavages of the mouse egg (adapted from Gulyas, 1975). (a) Hemispheric section of a mouse egg through the second polar body. The section lies along the animal–vegetal (A–V) axis. The future embryonic–abembryonic axis lies orthogonally. The site of sperm fusion is shown establishing the plane of first cleavage. (b) Two-cell embryo showing the second polar body close to the first cleavage plane and the A–V axis. (c) The position after the first half blastomere has undergone cleavage. (d) Four-cell embryo after cleavage of the second half blastomere. This view preserves the original cleavage planes. However, the cleavage plane of the second half blastomere turns during cytokinesis so that the second cleavage planes lie at right angles to each other; shown in (e). In the rabbit, this rotation takes place in a preferred direction (see Gulyas, 1975). No data are available for rotation in other species.

All four are part of the multi-molecular *Brca1*-associated genome surveillance complex (BASC) that is involved in double-stranded DNA break repair (Wang *et al.*, 2000). The *Nbn* and *Brca1* homozygous mutants are lethal by late-blastocyst stage (Liu *et al.*, 1996; Zhu *et al.*, 2001), and *Rad50* and *Blm* produce visible

Table 1. Mouse homozygous null mutants that show a pre- or peri-implantation phenotype

Homozygous mouse mutant	Time of first appearance of aberrant phenotype	Reference
Rad51 RAD51 homolog (<i>Saccharomyces cerevisiae</i>)	E1.0	Tsuzuki <i>et al.</i> , 1996
Pfn1 Profilin 1	E1.0	Witke <i>et al.</i> , 2001
Sfrs3 Splicing factor, arginine/serine-rich 3 (SRp20)	E2.0	Jumaa <i>et al.</i> , 1999
Aplp2 Amyloid beta (A4) precursor-like protein 2	E2.0	Rassoulzadegan <i>et al.</i> , 1998
Plcb3 Phospholipase C, beta 3	Lethal at E2.5	Wang <i>et al.</i> , 1998
Atp6l ATPase, H ⁺ transporting, lysosomal (vacuolar proton pump) 16 kDa	E2.5	Inoue <i>et al.</i> , 1999
Tcl1 T-cell lymphoma breakpoint 1	E2.5	Narducci <i>et al.</i> , 2002
Incenp Inner centromere protein	Visible effects by E2.5	Cutts <i>et al.</i> , 1999
Racgap1 RacGTPase-activating protein 1	Observable defects at E3.0	Van den Putte <i>et al.</i> , 2001
Catna1 Catenin alpha 1 (α -E-catenin)	E3.5	Torres <i>et al.</i> , 1997
Trrap Transformation/transcription domain-associated protein	E3.5	Herceg <i>et al.</i> , 2001
Smn Survival motor neurone	E3.5	Schrank <i>et al.</i> , 1997
Txn Thioredoxin	E3.5	Matsui <i>et al.</i> , 1996
Nbn Nibrin (nbs1)	About E4.0	Zhu <i>et al.</i> , 2001
Ap1g1 Adaptor A protein complex AP-1, gamma 1 subunit (γ -adaptin)	About E4.0	Zizioli <i>et al.</i> , 1999
Madh2 MAD homologue 2 (<i>Drosophila</i>) (Smad2)	Effect on brachyury (T) at E4.0, die at E7.5	Heyer <i>et al.</i> , 1999
Pou5f1 POU domain, class 5, transcription factor 1 (Oct4)	Effect on ICM, E4.0	Nichols <i>et al.</i> , 1998
Raly hnRNP-associated with lethal yellow	E4.0	Michaud <i>et al.</i> , 1993
Mbtps1 Membrane-bound transcription factor protease, site 1	E4.0	Yang <i>et al.</i> , 2001
Tbn -pending Taube nuss	E4.0	Voss <i>et al.</i> , 2000
Bub3 Budding uninhibited by benzimidazoles 3 homologue (<i>S. cerevisiae</i>)	Normal to E3.5 but mitotic errors from E4.5	Kalitsis <i>et al.</i> , 2000

Continued.

Table 1. (Continued)

Homozygous mouse mutant	Time of first appearance of aberrant phenotype	Reference
<i>Cdh1</i> Cadherin 1 (<i>E-cadherin</i>)	About E4.5	Larue <i>et al.</i> , 1994
<i>Brca1</i> Breast cancer 1	Effects detectable by E4.5	Liu <i>et al.</i> , 1996
<i>Vav</i> Vav oncogene	E4.5–E5.0	Zmuidzinas <i>et al.</i> , 1995
<i>Grb2</i> Growth factor receptor-bound protein 2	E4.5–E5.0	Cheng <i>et al.</i> , 1998
<i>Cdc45l</i> Cell division cycle 45 homologue (<i>S. cerevisiae</i> -like)	Between E4.5 and 5.5	Yoshida <i>et al.</i> , 2001
<i>Itgb1</i> Integrin beta 1 (fibronectin receptor beta)	Normal at E4.5, degeneration by E5.5	Stephens <i>et al.</i> , 1995
<i>Yy1</i> Yy1 transcription factor	Implant but degenerate immediately, E5.0	Donohoe <i>et al.</i> , 1999
<i>Ahcy</i> S-adenosylhomocysteine hydrolase	E5.0	Miller <i>et al.</i> , 1994
0610007L03Rik RIKEN cDNA 0610007L03 gene (Srb7)	E5.0	Tudor <i>et al.</i> , 1999
<i>Refbp1</i> RNA and export factor bonding 1	E5.5	Xanthoudakis <i>et al.</i> , 1996
<i>Mdm2</i> Transformed mouse 3T3 cell double minute 2	E5.5	Montes de Oca Luna <i>et al.</i> , 1995
<i>Cse1l</i> Chromosome segregation 1-like (<i>S. cerevisiae</i>)	Disorganized and degenerated by E5.5	Bera <i>et al.</i> , 2001
<i>Smarcc1</i> SWI/SNF-related, matrix- associated, actin-dependent regulator of chromatin	E5.5	Kim <i>et al.</i> , 2001
<i>Gata6</i> GATA binding protein 6	E5.5	Morrissey <i>et al.</i> , 1998; Koutsourakis <i>et al.</i> , 1999
<i>Cul1</i> Cullin 1	E5.5	Dealy <i>et al.</i> , 1999
<i>Pafah1b1</i> Platelet-activating factor acetylhydrolase, isoform 1b, beta 1 subunit	E5.5	Hirotsune <i>et al.</i> , 1998
<i>Cenpa</i> Centromere autoantigen A	Observable from E5.5	Howman <i>et al.</i> , 2000
<i>Ccna2</i> Cyclin A2	E5.5	Murphy <i>et al.</i> , 1997
<i>Trsp</i> tRNA phosphoserine	Die shortly after implantation, E5.5?	Bosl <i>et al.</i> , 1997
<i>Rad51L1</i> Rad51-like 1 (<i>S. cerevisiae</i>)	E5.5	Shu <i>et al.</i> , 1999
<i>Lamc1</i> Laminin, gamma 1	E5.5	Smyth <i>et al.</i> , 1999
<i>Evx1</i> Even-skipped homeotic gene 1 homolog	E5.5	Spyropoulos and Capecchi, 1994
<i>Tsg101</i> Tumour-susceptibility gene 101	E5.5, fail to develop past E6.5	Ruland <i>et al.</i> , 2001

Continued.

Table 1. (Continued)

Homozygous mouse mutant	Time of first appearance of aberrant phenotype	Reference
Fgfr2 Fibroblast growth factor receptor 2	Between E5.5 and E6.5	Arman <i>et al.</i> , 1998; Haffner-Krausz <i>et al.</i> , 1999
Ppp2ca Protein phosphatase 2a, catalytic subunit, alpha isoform	Between E5.5 and 6.5	Gotz <i>et al.</i> , 1998
Rad50 RAD50 homologue (<i>S. cerevisiae</i>)	Abnormal by E6.0	Luo <i>et al.</i> , 1999
Rangap1 RAN GTPase activating protein 1 (~ fug1, RNA1)	Arrest at egg cylinder stage, E6.0	DeGregori <i>et al.</i> , 1994
Dsp Desmoplakin	E6.0–E6.5	Gallicano <i>et al.</i> , 1998
Blm Bloom syndrome homologue (human)	Late lethality, visible effects on size by E6.5	Chester <i>et al.</i> , 1998
Bmpr2 Bone morphogenetic protein receptor 2	Growth retarded and disorganized, E6.5	Beppu <i>et al.</i> , 2000
Xrcc1 X-ray repair complementing defective repair in Chinese hamster cells 1	Arrest at E6.5	Tebbs <i>et al.</i> , 1999
Btf3 Basic transcription factor 3	Die at E6.5	Deng and Behringer, 1995
Clns1 Chloride channel, nucleotide sensitive, 1a (ICln)	E3.5–E7.5	Pu <i>et al.</i> , 2000

These mutants were identified using PubMed searches (www.ncbi.nih.gov/PubMed). The time to the first appearance of the aberrant phenotype is shown for each entry. In most cases, the first appearance of the phenotype is associated with growth arrest or cell death. In a smaller number of mutants, the early phenotype is associated with slow or abnormal growth or a failure to hatch from the blastocyst.

E: embryonic day.

effects by E6.5 (Chester *et al.*, 1998; Luo *et al.*, 1999). All four are in the inventory of preimplantation genes, along with other components of BASC (*Msh2*, *Msh6*, *Mlh1*, *Mre11*, *Rfc1*, *Rfc2*, *Rfc4*). *Brca1* and *Brca2* are involved in maintenance of chromosome stability as well as DNA repair and recombination, cell-cycle control and transcription (Venkitaraman, 2002). The yeast *Saccharomyces cerevisiae* possesses a DNA damage repair network that has recently been characterized in considerable detail by mass spectrometry of the purified complex (Ho *et al.*, 2002). Components of this network overlap with that in mammals, providing structural support for the function of DNA damage repair in mice and humans.

Other early embryonic-lethal genes that may also be involved in DNA repair include *Rad51* and *Rad51l1* as well as *Xrcc1*, for which the null mutant produces complete developmental arrest at E6.5 (Tsuzuki *et al.*, 1996; Shu *et al.*, 1999; Tebbs *et al.*, 1999). *Xrcc1* is part of a complex involved in single strand repair that includes polynucleotide kinase (*Pnk*), DNA polymerase-B (*Pol*), and DNA ligase III (*Lig3*) (Whitehouse *et al.*, 2001). Again, all have transcripts in the preimplantation gene inventory. Early homozygous lethal mutants that are

also associated with genomic segregation and mitotic progression include *Aplp2*, two centromere proteins (*Incenp*, *Cenpa*), as well as the checkpoint protein *Bub3* (Rassoulzadegan *et al.*, 1998; Cutts *et al.*, 1999; Howman *et al.*, 2000; Kalitsis *et al.*, 2000). Other early lethal phenotypes are associated with chromatin modification (*Smarcc1*, *Ttrap*), mRNA splicing (*Smn*, *Sfrs3*) and RNA binding (*Raly*, *Refbp1*) (Michaud *et al.*, 1993; Xanthoudakis *et al.*, 1996; Schrank *et al.*, 1997; Jumaa *et al.*, 1999; Herceg *et al.*, 2001; Kim *et al.*, 2001).

These genes are likely to show early embryonic lethality because they play key roles in cells in general, the early embryo being merely the first opportunity for expression of the null phenotype. Of more interest in the context of developmental mechanisms are genes that produce selective effects, either on the trophectoderm or ICM. For example, three mutants, *vav*, *cdh1* (*E-cadherin*) and α -*E-catenin*, affect the trophectoderm but not the ICM. *Vav* immunoreactivity is found in the trophectoderm but not the ICM of E3.5 embryos and *Vav* (–/–) embryos fail to hatch at the blastocyst stage (Zmuidzinas *et al.*, 1995). *E-cadherin* and α -*E-catenin* are essential for the formation of tight junctions in the trophectoderm at the eight-cell stage

(Larue *et al.*, 1994; Torres *et al.*, 1997). By contrast, the transcription factor *Pou5f1* is expressed in the ICM and is essential for maintenance of its pluripotency (Nichols *et al.*, 1998), and *Grb2* and $\beta 1$ *integrin* play roles in its differentiation and maintenance (Stephens *et al.*, 1995; Cheng *et al.*, 1998). *Taube nuss* and *Gata6* are transcription factors that act on the epiblast: *taube nuss* by suppressing apoptosis (Voss *et al.*, 2000) and *Gata6* by acting in extra-embryonic cells to maintain the epiblast (Koutsourakis *et al.*, 1999). Other transcription factors that are homozygous lethal in mouse pre- or periimplantation development are *Btf3*, *Mdm2* and *Yy1* (Deng and Behringer, 1995; Montes de Oca Luna *et al.*, 1995; Morrissey *et al.*, 1998; Donohoe *et al.*, 1999) and two other mutants have a direct effect on transcription: *Madh2* (–/–) mutants show a premature rise in brachyury (T) expression at E4.0 (Heyer *et al.*, 1999) and *Mbtps1* is a membrane-bound transcription factor protease (Yang *et al.*, 2001) that produces a phenotype by E4.0. These data are evidence that silencing transcription factors produces preimplantation phenotypes.

Other approaches to gene silencing

It has been recognized for some time that insertional mutagenesis ('gene trapping') in embryonic stem cells provides a route for construction of knockout mice. However, it relies on random insertions to produce silencing of genes which must then be characterized. Attempts to optimize the approach for genes of developmental interest has focused on the use of signal trapping to identify secreted and membrane proteins (Skarnes *et al.*, 1995; Wiles *et al.*, 2000; Mitchell *et al.*, 2001). The rationale has been that membrane proteins and their ligands are likely to be important in development. Although this is true, it is also the case that many membrane and secreted proteins play no role in development, whereas key developmental proteins such as transcription factors have no signal sequence and will not be caught in a signal trap. A much more rapid route to screening for mutant phenotypes using gene silencing is likely to be RNA interference (RNAi). RNAi is one of a number of RNA silencing mechanisms used to eliminate defective mRNAs and defend organisms against genomic parasites (transposons and viruses; Zamore, 2002). RNAi using short sequences of double-stranded RNAs that contain the sequence of a specific gene frequently reduces or suppresses transcription, providing highly selective transcript knockdown. Importantly, there is strong evidence, using *c-mos*, *t-PA* and *Oct-3/4* as target species, that RNAi is effective at ablating gene transcripts in preimplantation development (Svoboda *et al.*, 2000; Wianny and Zernicka-Goetz, 2000; Kim *et al.*, 2002). RNAi has already greatly increased the speed of functional screening for mutant phenotypes in *Caenorhabditis elegans* (Zipperlen *et al.*, 2001; Ashrafi *et al.*, 2003; Kamath *et al.*, 2003) in which, as is now

the case in the mouse preimplantation embryo, the DNA sequences of a large number of candidate genes are known. The lack of full-length cDNAs for many UniGenes is not, in itself, an obstacle because of the shortness of the RNA sequences required for RNAi.

Despite the potential for high throughput screening using RNAi, there remains a case for narrowing an initial screen because of the large starting number of candidates (about 15 700). The most likely genes to be developmentally important are transcription factors, secreted proteins, surface receptors and signalling pathways. It is possible to undertake a computer-based screen for these proteins by integrating the preimplantation gene inventory with a keyword search in an appropriate database. Bioperl software has been used to integrate the results of a search of the NIH LocusLink database with UniGene-based libraries (J. L. Stanton, A. B. Macgregor and D. P. L. Green, unpublished). By way of example, a search in LocusLink on the keyword 'transcription factor' identifies a current total of 622 identified transcription factors for the complete mouse genome, of which 178 are expressed in the mouse preimplantation embryo. Interestingly, about 120 transcription factors have been identified for early *Drosophila* embryogenesis (Berman *et al.*, 2002), a similar order of magnitude. A search of LocusLink using the keyword 'kinase' identifies 871 kinases or kinase-related proteins, of which 308 are expressed in mouse embryos before implantation. It can be argued that selected use of RNAi starting from a large inventory of known genes is inherently more inclusive and potentially faster than signal trapping in embryonic stem cells as a means of screening for developmentally interesting phenotypes, particularly with the arrival of electroporation of double-stranded RNAs into preimplantation embryos (Grabarek *et al.*, 2002).

Looking ahead

One aim of this review has been to outline an approach using functional genomics to identify genes the expression of which determines patterning and differentiation in mouse preimplantation development. Although this inventory of genes is currently limited in its sequence lengths and annotation, two developments are under way that should greatly facilitate progress. The first is the increasing amount and quality of information about expressed sequence. The Mammalian Gene Collection (MGC) Program (2002) has signalled its goal of obtaining full-ORF (full-open reading frame) cDNA sequence and clones for each gene in humans and mice and the FANTOM Consortium is committed to expanding its coverage of the transcriptome in mice. Unfortunately, the time frame over which these efforts will be spread is not known. Mapping of mouse UniGenes onto the draft mouse genome is already under way and should help produce a rationalization of the mouse UniGene

database analogous to that obtained with the human UniGene database (Zhuo *et al.*, 2001). Mouse genes are increasingly well annotated and this improvement will feed back to generate a high-quality annotated catalogue of genes expressed in embryonic development in mice. HomoloGene is steadily expanding to identify human and other mammalian orthologues of mouse genes. Fly, nematode and zebra fish orthologues will also increasingly emerge, opening up the opportunity for comparative studies of early embryonic development.

The second facilitating development will be integrated efforts to identify signalling pathways. A major model initiative in this area is the Alliance for Cellular Signaling (AfCS), which aims to understand the signalling relationship between cellular inputs and outputs as completely as possible (<http://www.afcs.org>). AfCS has recently begun work on the extensive characterization of the mouse cardiac myocyte and the mouse splenic B cell. As neither of these types of cell expresses a developmental programme, there is a strong case for characterizing a well-defined mouse developmental system. The authors of this review propose mouse preimplantation development. Its advantages include development that produces a well-defined number of cells in a timetable that is consistent and short (4–5 days), development that is independent of external signals (which greatly simplifies analysis), a large inventory of expressed genes that are becoming increasingly annotated, and a developmental programme that can be targeted by RNA interference and characterized with DNA microarrays. Completion of the draft mouse, human and rat genomes will provide the additional opportunity to identify the *cis*-regulatory modules governing preimplantation gene expression as the 5' ends of mouse preimplantation genes become identified.

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