Develop. Growth & Differ., 35 (4), 365–370 (1993)

Development
Growth & Differentiation

Review

Gene Knockouts and Murine Development

(gene knockout/gene targeting/*Mus*/mammalian embryology/genetic redundancy)

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A considerable quantity of data has been generated using the technique of *in vivo* gene knockout in mice, much of which is of relevance to the developmental biologist. Null mutations in *Hox* genes at the 3′-end of the clusters create complex irregularities at the rostral end of the embryo, including defects in the middle ear and the large blood vessels, suggesting that *Hox* genes may be involved in pattern specification of these structures in addition to the anteroposterior axis. Null mutations in oncogenes either cause wide pleiotropic effects, or act in a restricted manner on the haematopoietic system. Null mutations in growth factors and related molecules cause failure of proliferation in restricted areas of the embryo in some cases, but have little phenotype in others. There is as yet no null mutation which supports the idea that growth factors are involved in mesoderm induction in mammals. A surprising variety of genes have no null phenotype, or one less severe than might have been previously predicted on the basis of their known function *in vitro* and pattern of expression. This leads to the possibility that genetic redundancy exists in development.

In the last three decades, the mouse has become a favourite experimental organism for developmental biology, largely due to improved techniques of *in vitro* embryo culture and its convenience for genetics and molecular biology. In the past five years, the technique of site-directed mutation *in vivo*, colloquially known as gene knockout, has given a further boost to the field, enabling investigators to create null mutations in virtually any cloned gene of interest. The purpose of this review is to describe the results of these efforts to date, and to ask what they have so far contributed to our knowledge of murine development. Technical aspects will not be covered, as these have been considered by other authors (3). Briefly, the technique consists of the transfection of an embryonic stem cell line with a DNA construct containing a mutated gene sequence and a selectable marker. Successful transfectants are selected using the marker, and the mutated gene sequence may recombine with its homologue in those cells. Once a recombinant cell line has been isolated, cells can be combined with disaggregated preimplantation mouse embryos, and allowed to develop to adult chimerae. Cross breeding of the chimerae may, if the introduced cells have entered the germ line, lead to mice homozygous for the mutation.

A limited amount of evidence had been provided for the existence of a Hox code in mammals

Hox cluster genes are the mammalian homologues of the homoeotic genes in *Drosophila*, and were isolated on the basis of a short region of homology, the homoeobox, which has been shown to be a DNA-binding domain (for review see 25). The expression pattern of homoeobox RNAs in the mouse is suggestive of a binary 'Hox code' operating at least in the hindbrain and branchial arch region, and presumably elsewhere (12). This hypothesis states that unique combinations of expressed *Hox* genes would specify unique positional levels in the antero-posterior (or more correctly rostro-caudal) axis. A similar code could operate in the proximo-distal axis of the limb. This idea is based on work in *Drosophila*, where such a code almost certainly exists (for review see 36). However, there has always been some doubt as to whether the same principles can apply in a cellular mammalian embryo as do in the acellular nuclear blastoderm of *Drosophila* (for example see 8). Some experiments of the artificial overexpression of *Hox* genes (17, 22) have been consistent with the existence of such a code, but are beyond the scope of this review.

The *Hoxa-1* gene, a homologue of the *labial* gene of *Drosophila*, is intriguing in that it produces

p.c.-post-coitum, DNA-deoxyribonucleic acid, RNA-ribonucleic acid, r-rhombomere

two distinct products during normal embryogenesis, one with homoeodomain and one without. After 8 days *p.c.* the latter predominates. Between 7.5 and 8 days *p.c., Hoxa-1* is expressed in the central nervous system and mesoderm up to anterior boundary of the fourth rhombomere (r4). After 8.5 days *p.c. Hoxa-1* expression retreats caudally, is last found in the gut at 11.5 days *p.c.* and disappears a day later. Lufkin *et al.* (21) created a null mutant which removes both homoeobox and non-homoeobox products. This results in a malformed inner ear and skull bones, defects in rhombomeres r4-r7, and the absence of some cranial ganglia. Chisaka *et al.* (6) removed the homoeodomain product only, giving ear defects, no rhombomeres, and poorly formed ganglia IX and X, with ganglia VII and VIII shifted rostrally.

The Hox code hypothesis would predict that removal of a specific *Hox* cluster gene would alter the specification of a rhombomere or group of rhombomeres. However, in this case, it appears that the whole rhombomere pattern is disrupted. Nevertheless, the parts of the central nervous system rostral to the hindbrain appear to develop normally. The effects of the two mutants are slightly different with respect to the rhombomeres, implying that some interaction may be occurring between the two products which governs rhombomere formation. Both products may be involved in patterning of the inner ear. Alternatively, the ear defect may be a pleiotropic effect resulting from the perturbation of the anteroposterior pattern. The rostral shift in the position of the ganglia VII and VIII is, however, more strictly according to the predictions of the Hox code hypothesis.

Chisaka and Capecchi (5) created mutants in the *Hoxa-3* gene, a homologue of *zen* and *pb* in *Drosophila*. This gene is normally expressed at 7.5 to 8 days *p.c.* in the presomitic mesoderm and ectoderm, the spinal cord up to level of the otic vesicle, and by 12.5 days *p.c.* is also found in the pharynx, aortic trunk, thyroid, cranial ganglia, and the viscera. The null mutants are athymic, aparathyroid, and have reductions in the thyroid and submaxillary glands, with complex throat, heart, arterial and craniofacial defects resulting in death from cardiopulmonary failure at birth. This gene seems to be involved in patterning of the aortic arch region rather than anterior-posterior axis, and to be necessary for the development of the thymus, parathyroid gland and craniofacial region in general.

Le Mouellic *et al* (20) created null mutants in *Hoxc-8*, homologue of the *Abd-A* gene in *Drosophila*. This gene is much more 5′ than those above, and its anterior boundary of expression is therefore more posterior. These mutants die within a few days of brith, and have some interesting defects in the skeletal system. For instance, the 8th pair of ribs is on, instead of below, the sternum, and a 14th pair of ribs appear on the 1st lumbar vertebra. There are also other slight anteriorizations. So far *Hoxc-8* provides the null phenotype most consistent with the predictions of the Hox code hypothesis, in that it seems to turn the 8th pair of ribs into a second set of 7th ribs, and make the 1st lumbar vertebra into a thoracic vertebra.

One conclusion which may be drawn from this limited set of experiments so far published, is that the *Hox* cluster genes could be involved in other morphogenetic processes in addition to axial positional specification. The possibility that *Hox* genes govern the patterning of the large blood vessels and inner ear must be considered. With respect to the Hox code hypothesis, the evidence of the gene knockouts is not entirely clear. With the exception of *Hoxc-8*, patterning defects tend to be in non-axial structures (inner ear or large blood vessels). This may be due to the fact that the genes so far knocked out are mostly at the extreme 3′ end of the *Hox* clusters, and may therefore not necessarily be involved in strictly axial patterning. It might be predicted that genes more 5′ in the *Hox* cluster will produce mutants more consistent with the Hox code hypothesis.

Growth factors and related secreted molecules are important for development but have not so far been demonstrated to be involved in mesoderm induction

Growth factors and their relatives have been known for nearly a decade to be expressed in early embryogenesis, and they have been postulated as controlling factors in development. Many have differential patterns of expression in embryogenesis, and a few are capable of inducing mesoderm or creating a second axis in the *Xenopus* egg (for review see 14). As these molecules belong to several different gene families, there is no unifying hypothesis for their action in the manner of the Hox code. However, work on *Xenopus* (14) strongly suggests that they are involved in mesoderm induction and perhaps indirectly in neural induction.

One of the most interesting growth factor families is the transforming growth factor type β (TGF-β) family with many members ranging across vertebrates and *Drosophila*. TGF-β1 is the archetype of the family and has an extremely wide range of

both proliferative and antiproliferative functions, being expressed in very localised areas in murine development (19). It synergises with activins in animal cap mesoderm induction assays, but is not itself a mesoderm inducer (31). TGF-β1 null mutants die at 20 days after birth of wasting and inflammation (34). This gene thus seems to have no essential role in mesoderm induction in mouse, nor indeed in any developmental process outside of the immune system.

Inhibin α is also a member of the transforming growth factor type family forming heterodimers with the related activin β_A and β_B chains. Its null mutants have no inhibins ($\alpha\beta_A$ or $\alpha\beta_B$) but do have activins ($\beta_A\beta_A$ and $\beta_B\beta_B$). Inhibin has been postulated to have a role in the induction of the nervous system, since dominant negative activin receptor mRNA gives ectopic development of nervous tissue in place of ectoderm in *Xenopus* (11), and inhibins are natural antagonists of activins. However, it seems that inhibin α has no essential role in neurogenesis in the mouse. The null phenotype is the development of rapidly fatal gonadal tumours from four weeks of age (24).

Wnt-1 is the archetype of a large family of cell signalling molecules (for review see 27) and can create a double axis when injected into a *Xenopus* egg, but is not expressed early enough in frogs to be an endogenous axis-specifying molecule. In the mouse, it is expressed in mid-embryogenesis in the hindbrain and spinal cord. Two groups have performed knockouts in *Wnt-1*, the null phenotypes suggesting that it is necessary for the development of the hindbrain. One experiment resulted in the absence of midbrain and rostral hindbrain, with no midbrain or cerebellum at birth, and death within 24 hours (28), and the other gave mutants which have a little cerebellum. These are viable but severely ataxic (37). The area which is absent is composed of engrailed-expressing cells which are progressively deleted as development procedes (29).

Fgf-3 is a member of the fibroblast growth factor family, which is almost certainly important for mesoderm induction in amphibians (1). It is normally expressed in the primitive streak, rhombomeres r5 and r6 and the parietal endoderm. In later embryogenesis it is found in the cerebellum, retina, teeth and inner ear. The null mutants have fused or abnormal tail vertebrae, gross inner ear deficiencies, and die shortly after birth. Like the 3′ *Hox* cluster genes, it seems to be involved in ear development (23). There is, however, no defect in early mesoderm induction.

Gene knockout experiments on growth factors have so far produced rather disappointing phenotypes in mice, given their interesting patterns of expression in development. Where null phenotypes have been generated, these are perthaps more consistent with a role in growth control or patterning, rather than early induction of mesoderm or neural tissue. However, the great variety and number of genes within growth factor gene families means that a growth factor with an important role in early inductive events may yet be isolated.

Oncogene null phenotypes indicate a diversity of roles in development

A similarity has long been noted between cancerous and embryonic cells, resulting in much interest in the possible roles of oncogenes in development. Oncognes do not constitute a single gene family, and several different kinds of genes, including nuclear transcription factors and cytoplasmic protein kinases, come under this heading.

N-*myc* is a good candidate for an important role in development with expression in the primitive streak and mesoderm, then central nervous system and neural crest derivatives, and later in the viscera. The null mutants die as their hearts cease development at 9 days *p.c.* Other organ systems are also affected to a lesser degree (4).

c-*fos* is fairly ubiquitous in expression during embryogenesis. Its null phenotype is reduced weight and a 60% reduction in viability at birth. From 11 days after birth the mutants develop osteopetrosis (overgrowth of bone), failure of tooth and gamete development and poor lymphopoiesis. There is also a behavioural defect manifest as hyperactivity with reduced responsiveness, but balance and co-ordination are normal. (15, 39).

c-*src* (35) also gives rise in the null phenotype ot osteopetrosis with resulting reduced size and domed head. The mutants also have a lighter coat, no incisors and eyes blocked with mucous secretion.

Two oncogenes appear to be specifically involved in proliferation of the blood cells. The nulls die as a consequence of this and show little other pathology. These are c-*myb* (4), whose nulls have no erythropoiesis and die at 15 days *p.c.* and c-*abl* (38) whose null phenotype results in thymic and splenic atrophy.

Oncogene null phenotypes demonstrate that earlier speculations concerning the importance of these genes in development were justified. The

complex pleiotropy of some of the nulls may indicate that these genes are involved in several developmental processes, at both early and later stages of embryogenesis.

Miscellaneous null phenotypes

myf-5 is the earliest gene expressed in the myogenic pathway. Its null phenotype consists of delayed appearance of myotome. However, the embryos die at birth, not due to myotomal abnormalities but due to a defect in the sclerotome of the ribs (2). The related *MyoD* gene, which can turn fibroblasts into myoblasts in transfection experiments, and was therefore a good candidate for a principal regulator of myogenesis, does not seem to be necessary for development (32). Gene replacement has not yet managed to identify a gene necessary for onset of myogenesis.

En-2 (16) is one of a small family related to *Drosophila engrailed,* and is expressed in two bands in the hindbrain. The null phenotype results in very slightly altered cerebellar folding.

RB-1 is mutated in dominant retinoblastoma, and is involved in cell cycle regulation. The null phenotype consists of abnormal haematopoiesis, cell death in hindbrain and spinal ganglia (but no effect on the retina), with death at 14 to 15 days *p.c.* (7, 13, 18).

It is interesting that the *RB-1* mutation seems to generate a phenotype similar to *Wnt-1*, whereas its expected effect might be of retinal hyperproliferation. The *En-2* result is particularly interesting in the context of discussion of genetic redundancy (see below), given that its pattern of expression is conserved across all vertebrates so far examined, and therefore must have persisted for some hundreds of millions of years (9).

A surprising variety of genes demonstrate no null phenotype

One of the most surprising things to have emerged from these studies is the fact that many genes which were good candidates for a crucial role in development exhibit no visible defect in the null mutant. The following is an incomplete list, due to the fact that experiments resulting in no null phenotype are often thought less worthy of publication.

MyoD (see above) does not seem to be necessary for development (32).

None of the three retinoic acid receptors so far cloned in mouse seems to have a visible null phenotype (T. Lufkin, report to 5th Head Group Conference, London 1992), despite extensive differential patterns of expression in embryogenesis (10).

Tenascin, an extracellular matrix protein, which seems to be involved in heart development and other embryogenic processes, also has no null phenotype (33).

This data, combined with the unexpectedly mild phenotypes of null mutants for TGF-β1 and En-2 among others, has fuelled speculation concerning genetic redundancy in development (26), and this area is worthy of the attention of evolutionary theorists.

There is a limited amount of evidence that other genes may compensate for loss of function in the mutants

Where genes have no null phenotype, or the null phenotype is less severe than expected, investigators frequently speculate as to the possibility that the missing function of the gene is somehow compensated for by the activity of a related gene. This neglects the important point that members of gene families generally have widely differing patterns of expression. For instance, on the basis of expression patterns of TGF-β genes (30), it is difficult to see how TGF-β2 or β3 could compensate for the loss of TGF-β1. However, there is a small amount of evidence that suggests that some form of compensation may occur in some gene families. For instance, *MyoD* mutants have elevated *myf-5* mRNA in skeletal muscle which may compensate in part for the loss of myogenic function (2). *En-2* mutants only manifest the phenotype where *En-1* is not expressed (ie. in the cerebellum), suggesting that in the area where they are co-expressed *En-1* is able to compensate for the absence of the other (16). Likewise, the *Wnt-1* null phenotype is only found where no *Wnt-3a* is expressed (29). In N-*myc* mutants, the appearance of the phenotype begins at the stage when N-*myc* and c-*myc* are no longer con-expressed, suggesting that a similar process may be occuring in the *myc* gene family (4). If these few examples can be found to have parallels in other mutants, then it raises the question of whether some kind of genetic homoeostasis is occurring in the genome. However, the real explanation is likely to be much more prosaic, namely that gene family products exert negative regulation on each other, so that the removal of one member causes the others to be up-regulated. The compensatory response would therefore be co-incidental rather than adaptive.

Conclusions and further directions

The generation of null mutants has raised as many questions as it has provided answers. Among the most important may be ranked the following. If there really redundancy within the genome? If so, how is it maintained without the accumulation of mutations leading to formation of pseudogenes? What is the cause of asymmetry in the expression of some mutant phenotypes, particularly when the asymmetry is non-random? (21, 23).

On the other hand, enough data has already been generated to allow us to make reasonable generalisations concerning the roles of *Hox* cluster genes, oncogenes and growth factors in development. It is fair to say that *Hox* cluster genes are turning out to be even more interesting than was previously imagined, while growth factors have so far been a little disappointing. Oncogenes present some intriguing null phenotypes, demonstrating that they are involved in diverse aspects of development.

Future efforts will lead to the generation of many more null phenotypes which may confirm or refute these initial speculations. Another interesting possibility involves the generation of double mutants, although the variability of genetic background in the various mouse strains utilised means that this is far from a trivial prospect.

Acknowledgements: The author is supported by the Wellcome Foundation, and thanks colleagues and an anonymous reviewer for helpful comments on the manuscript.

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(Received April 19, 1993)