Cytogenet Genome Res 117:231–239 (2007)
DOI: 10.1159/000103184

Cytogenetic and Genome Research

The chicken as a model for embryonic development

M.G. Davey[a] C. Tickle[b]

[a]Division of Genetics and Genomics, Roslin Institute, Roslin Biocentre, Midlothian and
[b]Division of Cell and Developmental Biology, College of Life Sciences, University of Dundee, Dundee (UK)

Manuscript received 17 August 2006; accepted in revised form for publication by I. Nanda, 28 November 2006.

Abstract. The traditional strength of chicken embryos for studying development is that they are readily manipulated. This has led to some major discoveries in developmental biology such as the demonstration that the neural crest gives rise to almost the entire peripheral nervous system and the identification of signalling centres that specify the pattern of structures in the central nervous system and limb. More recently with the burgeoning discovery of developmentally important genes, chicken embryos have provided useful models for testing function. Uncovering the molecular basis of development provides direct links with clinical genetics. In addition, since many genes that have crucial roles in development are also expressed in tumours, basic research on chickens has implications for understanding human health and disease. Now that the chicken genome has been sequenced and genomic resources for chicken are becoming increasingly available, this opens up opportunities for combining these new technologies with the manipulability of chicken embryos and also exploiting comparative genomics.

Chicken embryology

Outline of chick embryo development

When a hen's egg is laid, development is already well underway and a two-layered disc of many thousands of cells lies on top of the yolk (Bellairs and Osmond, 2005). The series of events that take place during the next day or so, when the egg is incubated, sets up the body axes and defines where the various organs will form. The first visible sign that marks the head to tail axis of the future embryo is the primitive streak, a thin opaque band of cells that extends from the edge of the embryonic disc (PS, Fig. 1A). One of the first organ systems to develop is the vascular system. Blood islands are seen soon after one day of incubation and the circulation is established about a day later. All this time the embryo is growing and changing shape. The major regions of the embryo become recognizable, e.g. head, trunk and tail, followed by formation of specific organs such as limbs, eyes, lungs etc. during the third and fourth days of incubation (Fig. 1B). During the final stages of development, from ten days until twenty or twenty-one days when the chick hatches, there is considerable growth and also elaboration of differentiated cells and tissues including ossification of the skeleton and formation of feathers (Fig. 1C). The sequence of chick development has been described as an illustrated series of developmental stages by Eyal-Giladi and Kochav (1975) for the first seven hours of development and by Hamburger and Hamilton (1951) for development up to 20 days, allowing standardisation between researchers working on chick development. Furthermore the detailed description of the developing anatomy of the chick embryo in 'The Atlas of Chick Development' which builds on the Hamburger and Hamilton description of chick development (Bellairs and Osmond, 2005) has allowed reliable reporting of manipulated chick embryonic anatomy.

Request reprints from Cheryll Tickle
Division of Cell and Developmental Biology, College of Life Sciences
University of Dundee, Dundee, DD1 5EH (UK)
telephone: +44 1382 385 817; fax: +44 1382 385 386
e-mail: c.a.tickle@dundee.ac.uk

1424–8581/07/1174–0231$23.50/0

Classical experimental embryology

Major insights into developmental mechanisms have come through the ease of ablating tissues and of making tissue grafts in early chick embryos. Thus one can cut out a small piece of tissue from an embryo and then graft it to another site in the same embryo or to another embryo. Such microsurgery can be carried out through a window made in the shell, while the embryo is still lying in the egg. Experiments can also be performed on early chick embryos placed in simple culture systems although, in these systems, the embryos can only continue developing for short periods of time.

Identifying cell-cell interactions

One of the most challenging problems in embryonic development is to understand the mechanisms that ensure that all the parts of the body form in their proper places. Waddington's classical experiment on chick embryos in the 1930s (reviewed in Stern and Conrad, 2000) identified an organizer region – equivalent to the organizer that had recently been discovered in amphibian embryos – which specifies the body plan. This region is the knot-like structure at the tip of the primitive streak known as Hensen's node (HN, Fig. 1A), which when grafted ectopically in early chick embryos, induces the formation of a secondary body axis. This discovery was of major significance not only in terms of understanding how the body plan is laid down in the chick embryo but also because it illustrated that similar mechanisms operate in different vertebrates. Indeed Waddington then went on to demonstrate that mammalian embryos also had an organizer by grafting the node of a rabbit embryo ectopically in an early chick embryo and showing that it too could induce a secondary axis.

Other crucial long range signalling centres that specify the pattern of structures that develop in particular regions of vertebrate embryos were also first identified through grafting experiments in chicks. These include notochord/floor plate which specifies the pattern of neurons that develop with respect to top to bottom (dorso-ventral) of the neural tube (van Straaten et al., 1988, Yamada et al., 1991), the polarizing region of the limb bud which specifies the number and pattern of digits (Saunders and Gasseling, 1968), and the isthmic organizer, at the midbrain/hindbrain boundary which specifies the pattern of the adjacent regions of the brain (Marin and Puelles, 1994; reviewed in Wurst and Bally-Cuif, 2001).

Experiments in chick embryos have also demonstrated the existence of a myriad of very local cell-cell interactions between adjacent tissues. An example of this type of local interaction occurs between epithelial and mesenchymal tissues at many different times and places in the embryo and epithelial-mesenchymal interactions are often both dynamic and bidirectional. The discovery of such interactions stemmed again from the ease of locally ablating epithelial tissues in chick embryos. It is also possible to cleanly separate epithelial and mesenchymal tissues, then make recombinations, between, for example, the two tissues in different orientations or tissues from embryos at different stages of development and graft the recombined tissues back into the embryo. Thus, for example, in the developing chick limb bud, ablation experiments revealed that interactions between the apical ectodermal ridge (the epithelial thickening that rims the bud) and underlying mesenchyme are necessary for outgrowth (Saunders, 1948; Summerbell, 1974), while limbs developing from recombinations between a mesenchymal hull from a right wing bud and ectodermal jacket from a left wing bud demonstrated that interactions between the ectoderm (epithelium) covering the sides of the bud and the underlying mesenchyme control the development of the structures on the upper and lower sides of the limb (MacCabe et al., 1974).

Tracing cell fate

Increasingly sophisticated cell labelling techniques have been used to trace cell fate and to follow the movements of cells in chick embryos, again uncovering general principles applicable to all vertebrate embryos. Chick/quail chimeras were first recognised in the late 1960s as powerful tools for tracing cell fate and a whole raft of discoveries has come from their analysis (reviewed in Le Douarin, 2005). Such chimeras are made by grafting fragments of tissue from quail embryos into chick embryos and then following the fate of the grafted cells. Quail cells were traditionally recognised in chick embryos through differences in staining properties, but, more recently, quail-specific antibodies have been used. Chick embryos with such interspecific grafts are able to survive and even go on to hatch because the operations are carried out before the immune system has developed.

Chick/quail chimeras have, for example, led to the elucidation of the fate of cells from the neural crest, including the discovery that the crest gives rise to almost the entire peripheral nervous system. The neural crest is a small population of cells that arises at the edges of the neural plate. When the neural plate rolls up to form the neural tube, neural crest cells come to lie on top of the tube beneath the ectoderm. By tracing the fate of quail cells within chick embryos, it became clear that neural crest cells disperse along particular tracts within the embryo and give rise to a wide range of tissues, not only to the dorsal root and enteric ganglia, but also to the pigment cells of the skin, Schwann cells and, in the head, connective tissues. Most of the skull is derived from neural crest (reviewed in Creuzet et al., 2005). More recently, mouse/chick chimeras have been used to study the derivatives of the neural crest (Fontaine-Perus and Cheraud, 2005) and the development of somites (reviewed in Fontaine-Perus, 2000). Somites are segmented blocks of cells running down the back of the embryo on either side of the spinal cord and give rise to back muscle (and the muscle of the limbs and tongue) and vertebrae.

Another method of tracing the fate of cells in embryos is by locally applying lipophilic dyes that label cell membranes. This method has been used in chick embryos to trace short term movements of cells, the dye eventually becoming diluted by cell division. Thus fate maps of embryos have been created during gastrulation, a process in which some cells

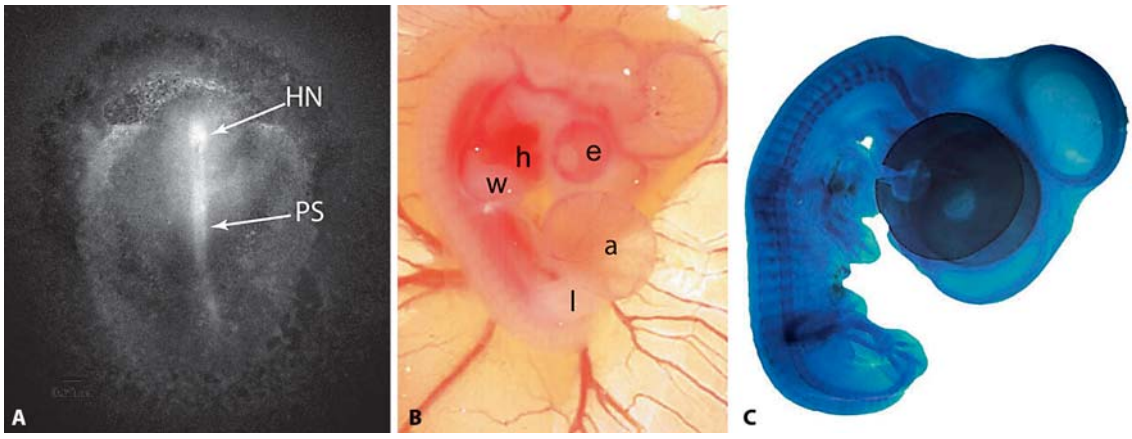

Fig. 1. Development of the chicken embryo. (**A**) Chicken embryo at the primitive streak stage of development after about 18 h of incubation. HN indicates Hensen's node, the organiser tissue in the early chick embryo; PS indicates the primitive streak. (Image from David Mcleod). (**B**) Chicken embryo in the egg, at stage 24HH, after four days of incubation. e = Eye, h = heart, w = wing, l = leg, a = allantois. HH = Hamburger-Hamilton stages (Hamburger and Hamilton, 1951). (**C**) Stained cartilage skeleton of a chicken embryo after 5–5.5 days of incubation (prior to ossification of the skeleton).

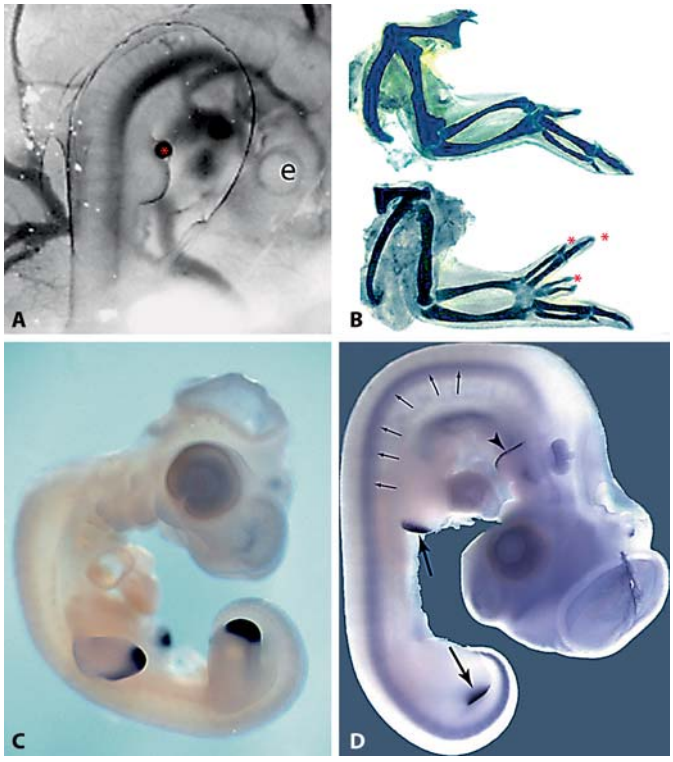

Fig. 2. Molecular basis of development. (**A**) Chicken embryo in the egg with a bead soaked in retinoic acid, grafted at the margin of the wing bud opposite the polarizing region; e = eye. (**B**) Stained wing skeleton of a normal wing (upper panel) with three digits, and a retinoic acid treated limb (bottom panel) with extra digits (red asterisk) which have been induced and are a 'mirror image' of the normal set of digits. (**C**) Expression of *HOXD13* in a four-day-old chicken embryo. The purple staining represents gene transcripts. *HOXD13* is expressed strongly at the tips of both wing and leg buds. (**D**) Expression of *SHH* in a stage 20HH chicken embryo. *SHH* is expressed at little finger/toe side of both wing and leg buds (arrow), in the face (arrowhead) and in the neural tube (small arrows).

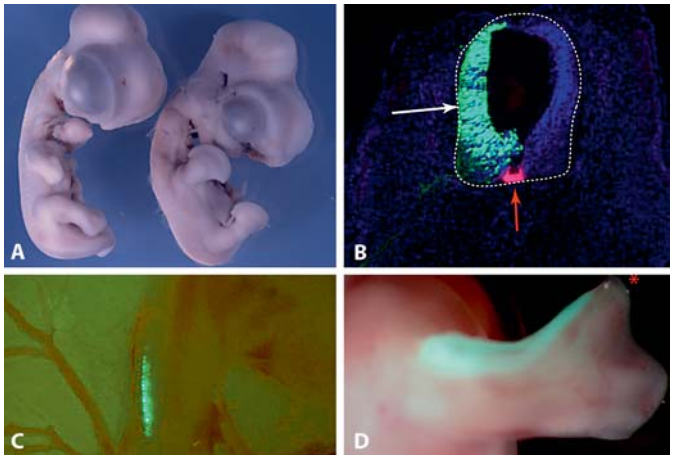

Fig. 3. Genomics in chick development. (**A**) A normal embryo (left) and a *talpid*^{3} embryo (right) after five days of incubation. Particularly noticeable are the large fan-like limbs of the *talpid*^{3} embryo. (**B**) A cross-section of a developing neural tube after three days of incubation. The neural tube has been electroporated with a GFP expression construct on the left side (white arrow) and left to develop for 24 hours. GFP is expressed in neurons and associated axons coming out of the left side of the neural tube (green) and the embryo has been subsequently stained using antibodies which label the floorplate cells (red cells indicated by red arrow). (**C**) An embryo expressing GFP controlled by a Sox2 promoter construct electroporated into the neural tube. The neural tube normally expresses Sox2 and this GFP construct is expressed in the neural tube in the same domain since its expression is controlled by Sox2 promoters. (Image from Bob Paton and Mike McGrew). (**D**) A limb of a normal embryo to which the polarizing region from an embryo from a GFP chicken line has been grafted to the opposite margin. The grafted polarizing region cells have induced extra digits in the limb (red asterisk) but since they are also permanently labelled with GFP, the contribution to the limb duplication can be readily seen in the living embryo and followed over time. (Image from Matthew Towers, graft by CT).

in the upper layer move to the primitive streak, then detach and move inside the embryo to create the three main body layers (e.g. Hatada and Stern, 1994). In addition, the fate of cells in older chick embryos in specific regions, such as the hindbrain (Fraser et al., 1990) or the developing limb (Altabef et al., 1997; Vargesson et al., 1997) has been traced using lipophilic dyes. Some of these experiments have revealed, rather surprisingly, the existence of cell lineage restricted compartments similar to those previously found in insects. In the chick hind brain, for example, the progeny of labelled cells are confined to just a single rhombomere (one of the seven recognizable hindbrain segments seen in both chick and mouse embryos) and do not straddle the boundaries with adjacent rhombomeres showing that each rhombomere constitutes a separate compartment (Fraser et al., 1990). Even more remarkably, the ectoderm in both limb and non-limb forming regions of the trunk of chick embryos was found to be compartmentalised with respect to the back and front of the embryo (Altabef et al., 1997). The compartment boundary running down the sides of the embryo appears to be important for positioning the limbs. Evidence for a similar boundary has also been discovered more recently in developing mouse limbs (Guo et al., 2003) showing that the initial findings in chick embryos are more widely applicable.

Labelling cells by expression of Green Fluorescent Protein (GFP) which can be visualised in living embryos has also been used to good effect in chick embryos (Fig. 3B, C, D). Gene constructs can be introduced into cells of chick embryos by electroporation or by using retroviruses (see later). In a recent set of experiments, primitive streak cells electroporated with GFP were cut out of one embryo and grafted into host embryos at different positions along the streak. Films of the movement trajectories of the labelled cells showed that the behaviour of streak cells depended on the position in which they were grafted, thus revealing the importance of local interactions in determining cell migration (Yang et al., 2002).

Molecular basis of development

Retinoic acid and cell-cell signalling

The first successful use of chick embryos to screen for molecules that can modulate development resulted in the finding that a vitamin A derivative, retinoic acid, could mimic signalling by the polarizing region in the limb bud. The signalling properties of the polarizing region, a small region of mesenchyme cells at the posterior margin of the limb bud, were first uncovered by grafting experiments (Saunders and Gasseling, 1968). When the polarizing region was cut out from the posterior margin of one chick wing bud and transplanted to the anterior margin of a second wing bud, an additional set of digits formed from the anterior part of the wing bud that normally does not form digits. This formed the basis for an assay to screen substances for polarizing activity (reviewed in Tickle, 2002). Unexpectedly, it was found that small pieces of paper or beads soaked in retinoic acid (Tickle et al., 1985; Fig. 2A) grafted to the anterior margin of chick wing buds could induce mirror-image duplications like those obtained following polarizing region grafts (Fig. 2B). There is now good evidence that endogenous retinoic acid plays a role in limb bud initiation and that, when applied to the anterior margin of a chick limb bud, triggers a cascade of expression of genes that encode cell-cell signalling molecules that control both digit number and pattern. It has also emerged that retinoic acid plays important roles in many other regions of vertebrate embryos including the central nervous system. Furthermore the use of small beads to apply locally defined molecules, including proteins and inhibitors, to chick embryos (Fig. 2A) has been exceptionally powerful in probing development.

Genes and development

Up until about 15 years ago, very little was known about the molecules that mediate cell-cell signalling and control cell fate in embryos. But since then there has been an explosion in discovering developmentally important genes through *Drosophila* mutants, tumours and clinical genetics. Although none of these genes was first identified in chickens, a new repertoire of manipulations devised to modulate gene expression and function has meant that chick embryos have been at the forefront in testing gene function.

A landmark in developmental biology was the discovery of the vertebrate Hox genes. These genes are relatives of the *Drosophila* homeotic genes which, when mutated, lead to rather grotesque phenotypes in which one part of the body is replaced by another. An example is the mutant Antennapedia in which an antennna is replaced with a leg. In insects, homeotic genes are clustered and arranged in sequence along the DNA (this cluster is split in two in *Drosophila*) and successive genes from the 3′ end of the cluster to the 5′ end are expressed in successive segments along the body of the insect from head to tail. The homeotic genes encode proteins that contain a DNA-binding domain and regulate expression of other genes and thus are responsible for determining the development of individual segments. In vertebrates, including chickens, there are four clusters of related Hox genes that appear to have undergone duplication during evolution. Furthermore as, in the fly, 3′ genes appear to be expressed at high levels towards the head end of the embryo while 5′ genes are expressed at higher levels towards the tail end (see Duboule and Dollé, 1989; Graham et al., 1989 for initial analysis in mouse embryos).

5′ members of two of the vertebrate *Hox* gene clusters are expressed in the early limb bud of both mouse and chick embryos in overlapping domains centred on the posterior tip of the limb where the polarizing region is located (Izpisúa-Belmonte et al., 1991; Fig. 2C) and the first evidence that these Hox genes are involved in patterning the limb came from experiments on chick embryos. Thus it was shown that manipulations that lead to formation of extra digits from anterior cells, polarizing region grafts or beads soaked in retinoic acid, also resulted in an ectopic pattern

of Hox gene expression anteriorly mirroring the pattern normally seen posteriorly. In addition, when one of the more 5′ *Hoxd* genes was inserted into a replication-competent avian-specific retrovirus which was then used to infect chick limb buds so that the gene was expressed throughout the limb bud, an additional digit formed and/or changes in digit morphology were induced (Morgan et al., 1992). These results have recently been illuminated by genetic manipulations in mice (Zakany et al., 2004).

Many other developmentally important genes, including genes encoding cell-cell signalling molecules, have been discovered through finding relatives of *Drosophila* genes. One of these vertebrate gene families comprises the three hedgehog genes, which are related to the *Drosophila hedgehog* gene and includes the *Sonic hedgehog (Shh)*. *Shh* encodes a long range signalling molecule which turns out to be involved in the development of virtually every part of a vertebrate embryo (reviewed in Ingham and MacMahon, 2001). Chicken embryos have played a key role in dissecting the function of Shh, beginning with the finding that Shh was expressed in several signalling centres in the chick embryo, including the polarizing region of the limb (Riddle et al., 1993; Fig. 2D), notochord and floor plate (Roelink et al., 1994; Fig. 2D), Hensen's node (Levin et al., 1995) and in the zona limitans intrathalamica (ZLI), at forebrain/midbrain boundary (Kiecker and Lumsden, 2004). Experiments in chick embryos showed that Shh, like retinoic acid, can act as a polarizing signal in the developing limb. When cells expressing Shh or beads soaked in Shh were placed at the anterior margin of a chick wing bud, mirror-image duplications of the digits developed (Riddle et al., 1993; Yang et al., 1997). Furthermore, it was shown that beads soaked in retinoic acid induced ectopic expression of Shh, thus providing a mechanism for the induction of digit duplications by retinoic acid (Riddle et al., 1993). Shh was also shown to mediate patterning of the neural tube by the notochord/floorplate and also to pattern the adjacent somites. Shh is expressed asymmetrically in the chick node and was one of the first genes to be shown to be involved in setting up Left/Right asymmetry through a signalling cascade including the secreted factors, Nodal and Lefty (Levin et al., 1995).

Another important way in which developmentally important genes were first identified is via tumours. Thus, for example, members of the fibroblast growth factor gene family such as *FGF4* were first identified as oncogenes expressed in human stomach tumours. *FGF4* is expressed in the posterior part of the apical ectodermal ridge of the limb bud and experiments in chick embryos demonstrated its role in apical ridge signalling. When the apical ectodermal ridge is cut away from the tip of the limb bud, limb truncations are produced. Outgrowth and patterning can be restored by stapling a bead soaked in Fgf4 protein to the tip of the limb bud in place of the apical ridge (Niswander et al., 1993). Another crucial family of genes encoding signalling molecules, the Wnt gene family was discovered through research on both tumours (int genes) and *Drosophila* (wingless) mutants.

Links with clinical genetics

Over the last few years, an increasing number of genes associated with human malformations have been identified by clinical geneticists. It has been very satisfying to see how these discoveries have converged with those coming from basic biological research. One of the first genes to be identified as being responsible for a congenital abnormality in human patients was a member of the *GLI* family of genes, so-called because a *GLI* gene was first identified in human glioblastomas. *GLI3* is the gene that is affected in patients with Greig cephalosyndactyly (Vortkamp et al., 1991) who typically have fused toes and an extra toe in front of the big toe. Embarrassingly for developmental biologists studying the limb, the function of *GLI3* was not understood at that time and there was no satisfactory explanation for the phenotype of the human patients. It soon emerged however that the *GLI* genes (which encode zinc finger transcription factors) are related to *Cubitus interruptus*, a gene in *Drosophila* whose product acts as the transcriptional effector of Hh signalling. Experiments in chick embryos showed that Shh signalling regulates expression of Gli1, Gli2 and Gli3 in the limb and that overexpression of an activated form of Gli1 could induce expression of Shh target genes (Marigo et al., 1996; Schweitzer et al., 2000). More recently it has emerged that Gli proteins (in particular Gli2 and Gli3) can act as both activators and repressors of Shh targets. Furthermore work on transgenic mice has shown that the action of the Gli3 repressor prevents digits forming in front (anterior) of the normal set of digits, thus providing an explanation for the extra toe seen in human patients with Greig cephalosyndactyly. It has also emerged rather unexpectedly from sophisticated genetic experiments in mice that the main function of Shh is to prevent the formation of Gli3 repressor and thus allow a patterned set of digits to form (Litintung et al., 2002; te Welscher et al., 2002).

Other genes that were discovered early on to be associated with human limb malformations included genes that encoded the receptors for FGFs and Hox genes. The pace of discovery in clinical genetics has been spectacular. In 1996 just seven genes associated with human limb defects were known, but by 2006 this number had risen to 37 (Ferretti and Tickle, 2006). It is also worth noting that studies on chick embryos have contributed to understanding the functions of many of these genes and thus provided insights into the mechanisms that lead to congenital malformations in human patients.

Genomics and chicken development

The recent breakthroughs in chicken genomic resources have reconfirmed the chicken as an exceptional model organism for developmental biology. Sequencing of the chicken genome has allowed forward genetics to be applied to a chicken developmental mutant for the first time. We are now able to easily identify chicken homologues of genes known to act during development in other species as well as to search for new developmentally regulated genes or regulatory elements. The comprehensive EST databases will enable in silico expression analysis and large scale microarray

based expression profiling thus uncovering the developmental regulation of gene expression on a genome wide scale. Transgenic technologies in chicken also now allow manipulation of gene expression either transiently or stably during development.

Forward genetics

Detailed maps of the chick genome have recently allowed, for the first time, forward genetic analysis of a chicken developmental mutant, *talpid*^{3}, and led to the identification of KIAA0586 as being the affected gene (Davey et al., 2006). In many ways, the *talpid*^{3} story highlights the way in which the explosion of genomic information has complemented the cut and paste era of developmental biology. *Talpid*^{3} is a recessive mutation which has been a focus for developmental biologists due to its extreme phenotype (Ede and Kelly, 1964). *Talpid*^{3} embryos have large polydactylous limbs, (limbs with many digits) which have no digit identity (Fig. 3A), holoproscencephaly (a loss of midline structures in the face and head), and die after only 3–7 days of embryonic development due to severe vascular abnormalities (Davey et al., 2007). The accessibility of the chicken mutant to surgical manipulations such as recombination experiments between *talpid*^{3} and wild-type limb bud tissue has shed light on the mutant phenotype. Grafting experiments have shown that the *talpid*^{3} tissue throughout the limb bud has 'polarizing' activity as any part of the *talpid*^{3} limb bud could induce extra digits when grafted into wild-type limb buds (Francis-West et al., 1995). However expression of Shh, which is often associated with abnormal polarizing activity, is normal and furthermore the *talpid*^{3} limb appears to be 'immune' to limb manipulations which target the Hedgehog (Hh) pathway, such as adding Shh soaked beads and/or inhibitors of Shh, or even chopping out the Shh expressing cells (Lewis et al., 1999; Davey et al., 2006). Analysis of single stranded conformational polymorphisms (SSCP) also showed that the *talpid*^{3} mutation did not lie in several of the known components of the Hh pathway (Lewis et al., 1999).

With the growth in chick genomic resources, mapping the *talpid*^{3} mutation became possible. Initial analysis of microsatellite markers between *talpid*^{3} in-bred carriers and non-carriers compared to the East Lansing Map indicated that the mutation lay in a region of chicken chromosome 5. The challenge was then to reconstruct a detailed physical map of the area by reference to the orthologous human region on chromosome 14, and, using known chicken gene sequences and sequencing BAC clones and ESTs, to develop a map of high density PCR-based markers which would show size, SSCP or single nucleotide polymorphism (SNP) variation in genes in the interval of interest. This approach was successful and a frame-shift mutation was uncovered in the sequence of the KIAA0586 gene, which would be predicted to result in a truncated transcript. To prove that this mutation is responsible for the *talpid*^{3} phenotype, the wild-type KIAA0586 gene was then placed back into cells of *talpid*^{3} embryos using in ovo electroporation and this was shown to rescue specific aspects of the mutant phenotype. KIAA0586 is a novel gene, predicted to consist of 1,524 aa and with no specific sequence motif which would place it within a gene family or indicate its function. Therefore all that we know of its function has been deduced from experiments in the *talpid*^{3} chicks which include histological data, surgical manipulative experiments and gene expression data. Based on this and comparison with Hh pathway mutants in mice and zebrafish, we hypothesised that the talpid3 protein acts in the transduction of the Hh signal (Lewis et al., 1999) at the level of the Gli transcription factors. Recent experiments have shown that the Gli3 transcription factor is not processed normally in *talpid*^{3} chick cells and that the *talpid*^{3} neural tube phenotype can be rescued by transfecting *talpid*^{3} cells in ovo with an activated Gli3 construct (Davey et al., 2006) confirming the role of talpid3 in Hh signal transduction.

Use of genomic resources – gene expression profiling

Although mapping of chicken mutations is important, it is not a day to day occurrence in most chick developmental biology labs, but the new genomic resources offer other possibilities to developmental biologists. Sequenced and annotated EST libraries (Boardman et al., 2002) should allow analyses of the expression profiles of embryonic tissues to be undertaken in silico. EST databases can be the first step in examining gene expression during development and offer some information about the expression pattern of a gene. They are also often called upon for sequence information to retrieve or identify the chicken sequence of interest and are also widely used in order to obtain EST clones to make RNA probes to examine gene expression by RNA in situ hybridisation in the embryo.

The prospect of realising the full power of chicken ESTs in developmental biology will be in microarray experiments. Whether used in a custom made microarray or in the Affymetrix microarray, developmental biologists now have the opportunity to examine expression of over 33,000 genes in one experiment. In the past, developmental biologists have only been able to examine expression of a limited number of genes, either because of the limitations on experimental sample number, or because of the limited number of genes known to act in chick development. Now many chick researchers will be able to re-examine their experimental system and classical experiments in order to uncover new genes and gene networks affected by their manipulations. Work on chicken embryos will yield particularly specific data about experimental systems that will not be easy to examine in other species due to the ease that chicks can be manipulated, the generous sample size this can yield, as well as the quality of precise timing and range of experiments that can be undertaken. The current embryological framework and knowledge of gene function in the chick should aid in designing microarray experiments and interpreting the wealth of new data. Furthermore experimental data generated by different chick experiments, even in different developmental systems, will be able to be compared to expose the genes involved in development and their precise regulation.

Use of genomic resources – uncovering important sequences

The chick genome has fewer, smaller genes and fewer pseudogenes than *Xenopus laevis*, zebrafish, mouse or human. This not only makes it a good anchor for the analysis of other genomes but it also makes it simpler than in other species to detect true homologues of developmentally important genes and to manipulate their expression (see transgenesis below).

The increase in available chick sequence and the completion of the genome has allowed the search for novel members of families of genes which are important in development. For example, chicken *Tbx18* was initially discovered through BLASTing the Tbx motif against the UMIST EST collection and has been since found to be important in somite, limb and heart development (Tanaka and Tickle, 2003).

Comparison of the chicken genome with other genomes has also revealed conserved regions of the genome that act as regulatory elements for gene expression in specific spatio-temporal patterns during development. Beckers et al. (1996) compared the sequence of the *Hoxd-11* loci in chicken, mouse and zebrafish sequence and uncovered one conserved region which controlled the expression of *Hoxd-11* during development, when tested in the mouse limb. In comparison Lin et al. (2006) compared the regulatory sequences of chicken genes which showed a transcriptional response to Shh in a microarray expression profile of chicken feather bud development. They found in these genes a common transcriptional responsive element which is also present in mouse and human and which was then found to be required for expression of the targets of the Hh signalling pathway in a chicken cell line. And yet other groups have combined the identification of chicken regulatory sequences with transgenesis in order to dissect the spatio-temporal action of different enhancers during development (Uchikawa et al., 2003; see transgenesis below). Furthermore it has only been realised since the large scale sequencing of the developmental specific EST libraries that there are a large (as yet undetermined) number of microRNAs (miRNAs) which have developmentally specific expression patterns (Hubbard et al., 2005). miRNAs regulate gene expression at the level of the transcript and the chick has already been instrumental in determining how they act in development, for example that miR-196 regulates expression of *Shh* and *Hoxb8* (Hornstein et al., 2005) and that miR-206 is regulated by Fgf signalling in myogenesis (Sweetman et al., 2006). The chick can also be used to investigate the expression patterns of miRNAs during development through the use of LNA-modified probes (Darnell et al., 2006; Sweetman et al., 2006). Furthermore advances in identifying miRNAs have opened up further potentials for knocking down gene expression in the chick (see below).

Reverse genetics and transgenesis

Transient transgenesis is now a routine experimental procedure in chick developmental biology. While over-expression of genes via a replication competent viral vector allows continued expression of the gene of interest throughout development, expression does depend upon efficient infection of the embryo by the virus. In order to overexpress genes transiently in chick embryos during development, electroporation is now more commonly used to directly transfect expression vectors of interest (including proviral expression vectors) into appropriate tissues. Electroporation is particularly good at targeting epithelial tissues such as the epiblast or hypoblast of the early embryo, the neuroepithelium (Fig. 3B) and somites (although other tissues have been successfully targeted, Eblaghie et al., 2003) and has been used to study the overexpression of genes such as *Pax5* in the developing brain (Funahashi et al., 1999) and the role of Gli proteins in the neural tube (Stamataki et al., 2005).

Electroporation has also been used to characterise functionally *cis*-acting enhancer and promoter elements. In these assays, a construct containing the putative enhancer element placed upstream of a minimal promoter driving a reporter gene is electroporated into the developing embryo and enhancer activity is shown by the reporter (Fig. 3C). This has been used in chick embryos to study mouse enhancers, as a fast and cheap alternative to making a transgenic mouse (Timmer et al., 2001) and has also been used to examine the spatiotemporal control of *SOX2* expression in the chick neural tube by a combination of enhancer elements (Uchikawa et al., 2003).

Until recently, knock-down strategies in the chick have not been very successful but new techniques based on RNA-interference (RNAi) coupled with electroporation have made loss-of-function studies possible. Several methods exist to modify gene expression through RNAi, some targeting the gene of interest through chemically synthesised short interfering RNAs (siRNA) or long double stranded RNAs (dsRNA; Pekarik et al., 2003). A recently developed tailor-made chick vector comprises a chicken promoter, a Red Fluorescent Protein (RFP) reporter and miRNA sequences to synthesize two short hairpin RNAs (shRNA) with the target sequence of interest (which should then be processed by RNAi machinery to produce siRNA sequences). It has been shown that this vector can be used to efficiently knock-down gene expression in the developing chick nervous system (Das et al., 2005).

Unlike other areas of development, the production of a stable transgenic chicken line has been hindered rather than helped by the special features of the chicken egg. The pronuclei and early blastocyst are not as easily accessible in the chick as they are in mouse because they are inherently attached to the yolk and white of the egg. Nevertheless, culture methods and lentiviral targeting via the subgerminal cavity of the early blastodermal embryo has produced stable ubiquitously expressing GFP and lac-Z chicken lines (McGrew et al., 2004). In particular the GFP line is a modern advance on cell tracking and labelling techniques as GFP cells can be grafted between donor and host in order to track cells with no loss of signal or potential cross-species problems seen with dyes or chick-quail chimeras (Fig. 3D). It has recently been shown that two cell types, chicken embryonic

stem cells derived from the blastocyst (CESCs) and primordial germ cells (PGCs; the precursors to eggs and sperm) can be easily isolated from embryos, cultured and then genetically manipulated and returned to developing embryos via the blood circulation, where they contribute to the developing embryo resulting in a chimera (reviewed in Sang, 2004). CECSs contribute to the somatic cells and in the future may be a useful way of examining gene function using chimeric embryos. Unlike CECSs, however, the PGCs preferentially contribute to the gonad and so the resulting chimeric chickens can readily produce stable transgenic lines (van de Lavior et al., 2006). This great advance in chicken transgenesis will hopefully herald an era of developmental biology which will see the production of transgenic chickens expressing tissue-specific transgenes as well as tissue-specific knock-downs through the stable expression of RNAi vectors.

References

Altabef M, Clarke JDW, Tickle C: Dorso-ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. Development 124:4547–4556 (1997).

Beckers J, Gerard M, Duboule D: Transgenic analysis of a potential Hoxd-11 limb regulatory element, present in tetrapods and fish. Dev Biol 180:543–553 (1996).

Bellairs R, Osmond M: The Atlas of Chick Development, 2nd Ed (Elsevier Academic Press, Oxford 2005).

Boardman P, Sanz-Ezquerro JJ, Overton I, Burt DW, Bosch E, et al: A comprehensive collection of chicken cDNAs. Curr Biol 12:1965–1969 (2002).

Creuzet S, Couly G, Le Douarin NM: Patterning the neural crest derivatives during development of the vertebrate head: insights from avian studies. J Anat 207:447–459 (2005).

Darnell DK, Simran K, Stanislaw S, Konieczka JK, Yatskievych TA, Antin PB: MicroRNA expression during chick embryo development. Dev Dyn 235:3156–3165 (2006).

Das RM, Van Hateren NJ, Howell GR, Farrell ER, Bangs FK, et al: A robust system for RNA interference in the chicken using a modified microRNA operon. Dev Biol 294:554–563 (2005).

Davey MG, Paton IR, Yin Y, Schmidt M, Bangs FK, et al: The chicken *talpid*^{3} gene encodes a novel protein essential for Hedgehog signaling. Genes Dev 20:1365–1377 (2006).

Davey MG, James J, Paton IR, Burt DW, Tickle C: Analysis of *talpid*^{3} and wild-type chicken embryos reveals roles for Hedgehog in development of the limb bud vasculature. Dev Biol 301: 155–165 (2007).

Duboule D, Dollé P: The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. EMBO 8:1497–1505 (1989).

Eblaghie MC, Lunn JS, Dickinson RJ, Munsterberg AE, Sanz-Ezquerro JJ, et al: Negative feedback regulation of FGF signaling levels by Pyst1/MKP3 in chick embryos. Curr Biol 13:1009–1018 (2003).

Ede DA, Kelly WA: Developmental abnormalities in the trunk and limbs of the talpid3 mutant of the fowl. J Embryol Exp Morphol 12:339–356 (1964).

Eyal-Giladi H, Kochav S: From cleavage to primitive streak formation; a complementary Normal table and a new look at the first stages of the development of the chick. I. General morphology. Dev Biol 49:321–337 (1975).

Ferretti P, Tickle C: The Limbs, in Feretti P, Copp A, Tickle C, Moore G (eds): Embryos, Genes and Birth Defects, 2nd Ed, pp 123–166 (Wiley, Chichester 2006).

Fontaine-Perus J: Mouse-chick chimera: an experimental system for study of somite development. Curr Top Dev Biol 48:269–300 (2000).

Fontaine-Perus J, Cheraud Y: Mouse-chick neural chimeras. Int J Dev Biol 49:349–353 (2005).

Francis-West PH, Robertson KE, Ede DA, Rodriguez C, Izpisua-Belmonte JC, et al: Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta3) limb buds: their relationships in the signalling cascade involved in limb patterning. Dev Dyn 203: 187–197 (1995).

Fraser S, Keynes R, Lumsden A: Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. Nature 344:431–435 (1990).

Funahashi J, Okafuji T, Ohuchi H, Noji S, Tanaka H, Nakamura H: Role of Pax-5 in the regulation of a mid-hindbrain organizer's activity. Dev Growth Differ 41:59–72 (1999).

Graham A, Papalopulu N, Krumlauf R: The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. Cell 57:367–378 (1989).

Guo Q, Loomis C, Joyner AL: Fate map of mouse ventral limb ectoderm and the apical ectodermal ridge. Dev Biol 264:166–178 (2003).

Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. J Morph 88:49–92 (1951); reprint Dev Dyn 195: 231–72 (1992).

Hatada Y, Stern CD: A fate map of the epiblast of the early chick embryo. Development 120:2879–2889 (1994).

Hornstein E, Mansfield JH, Yekta S, Hu JK, Harfe BD, et al: The microRNA miR-196 acts upstream of *Hoxb8* and *Shh* in limb development. Nature 438:671–674 (2005).

Hubbard SJ, Grafham DV, Beattie KJ, Overton IM, McLaren SR, et al: Transcriptome analysis for the chicken based on 19,626 finished cDNA sequences and 485,337 expressed sequence tags. Genome Res 15:174–183 (2005).

Ingham PW, McMahon AP: Hedgehog signaling in animal development: paradigms and principles. Genes Dev 15:3059–3087 (2001).

Izpisúa-Belmonte J-C, Tickle C, Dollé P, Wolpert L, Duboule D: Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. Nature 350: 585–589 (1991).

Kiecker C, Lumsden A: Hedgehog signaling from the ZLI regulates diencephalic regional identity. Nat Neurosci 7:1242–1249 (2004).

Le Douarin NM: The Nogent Institute–50 years of embryology. Int J Dev Biol 49:85–103 (2005).

Levin M, Johnson RL, Stern CD, Kuehn M, Tabin C: A molecular pathway determining left-right asymmetry in chick embryogenesis. Cell 82: 803–814 (1995).

Lewis KE, Drossopoulou G, Paton IR, Morrice DR, Robertson KE, et al: Expression of ptc and gli genes in talpid3 suggests bifurcation in Shh pathway. Development 126:2397–2407 (1999).

Lin SL, Chang SJ, Ying SY: Transcriptional control of Shh/Ptc1 signaling in embryonic development. Gene 367:56–65 (2006).

Litingtung Y, Dahn RD, Li Y, Fallon JF, Chiang C: Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature 418:979–983 (2002).

MacCabe JA, Errick J, Saunders JW: Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. Dev Biol 39:69–82 (1974).

Marigo V, Johnson RL, Vortkamp A, Tabin CJ: Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev Biol 180:273–283 (1996).

Marin F, Puelles L: Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. Dev Biol 163:19–37 (1994).

McGrew MJ, Sherman A, Ellard FM, Lillico SG, Gilhooley HJ, et al: Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep 5:728–733 (2004).

Morgan BA, Izpisúa-Belmonte JC, Duboule D, Tabin CJ: Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. Nature 358:236–239 (1992).

Niswander L, Tickle C, Vogel A, Booth I, Martin GR: FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. Cell 75:579–587 (1993).

Pekarik V, Bourikas D, Miglino N, Joset P, Preiswerk S, Stoeckli ET: Screening for gene function in chicken embryo using RNAi and electroporation. Nat Biotechnol 21:93–96 (2003).

Riddle RD, Johnson RL, Laufer E, Tabin C: Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75:1401–1416 (1993).

Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, et al: Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell 76: 761–775 (1994).

Sang H: Prospects for trangenesis in the chick. Mech Dev 121:1179–1186 (2004).

Saunders JW: The proximo-distal sequence of origin of parts of the chick wing and the role of the ectoderm. J Exp Zool 108:363–404 (1948).

Saunders JW, Gasseling MT: Ectodermal-mesenchymal interactions in the origin of limb symmetry, in Fleischmeyer R, Billingham RE (eds): Epithelial-Mesenchymal Interactions, pp 78–97 (Williams & Wilkins, Baltimore 1968).

Schweitzer R, Vogan KJ, Tabin CJ: Similar expression and regulation of Gli2 and Gli3 in the chick limb bud. Mech Dev 98:171–174 (2000).

Stamataki D, Ulloa F, Tsoni SV, Mynett A, Briscoe J: A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. Genes Dev 19:626–641 (2005).

Stern C, Conrad H: Waddington's contributions to avian and mammalian development, 1930–1940. Int J Dev Biol 44:15–22 (2000).
Summerbell D: A quantitative analysis of the effect of excision of the AER from the chick limb-bud. J Embryol Exp Morphol 32:651–660 (1974).
Sweetman D, Rathjen T, Jefferson M, Wheeler G, Smith TG, et al: FGF-4 signaling is involved in mir-206 expression in developing somites of chicken embryos. Dev Dyn 235:2185–2191 (2006).
Tanaka M, Tickle C: Tbx18 and boundary formation in chick somite and wing development. Dev Biol 268:470–480 (2004).
te Welscher P, Zuniga A, Kuijper S, Drenth T, Goedemans HJ, et al: Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. Science 298:827–830 (2002).
Tickle C: The early history of the polarizing region: from classical embryology to molecular biology. Int J Dev Biol 46:847–852 (2002).
Tickle C, Lee J, Eichele G: A quantitative analysis of the effect of all-trans-retinoic acid on the pattern of chick wing development. Dev Biol 109: 82–95 (1985).
Timmer J, Johnson J, Niswander L: The use of in ovo electroporation for the rapid analysis of neural-specific murine enhancers. Genesis 29:123–132 (2001).
Uchikawa M, Ishida Y, Takemoto T, Kamachi Y, Kondoh H: Functional analysis of chicken Sox2 enhancers highlights an array of diverse regulatory elements that are conserved in mammals. Dev Cell 4:509–519 (2003).
van de Lavoir MC, Diamond JH, Leighton PA, Mather-Love C, Heyer BS, et al: Germline transmission of genetically modified primordial germ cells. Nature 441:766–769 (2006).
van Straaten HW, Hekking JW, Wiertz-Hoessels EJ, Thors F, Drukker J: Effect of the notochord on the differentiation of a floor plate area in the neural tube of the chick embryo. Anat Embryol (Berl) 177:317–324 (1988).
Vargesson N, Clarke JDW, Vincent K, Coles C, Wolpert L, Tickle C: Cell fate and gene expression in chick limb. Development 124:1909–1918 (1997).
Vortkamp A, Gessler M, Grzeschik KH: GLI3 zinc-finger gene interrupted by translocations in Greig syndrome families. Nature 352:539–540 (1991).
Wurst W, Bally-Cuif L: Neural plate patterning: upstream and downstream of the isthmic organizer. Nat Rev Neurosci 2:99–108 (2001).
Yamada T, Placzek M, Tanaka H, Dodd J, Jessell TM: Control of cell pattern in the developing nervous system: polarizing activity of the floor plate and notochord. Cell 64:635–647 (1991).
Yang X, Dormann D, Munsterberg AE, Weijer CJ: Cell movement patterns during gastrulation in the chick are controlled by positive and negative chemotaxis mediated by FGF4 and FGF8. Dev Cell 3:425–437 (2002).
Yang Y, Drossopoulou G, Chuang P-T, Duprez D, Marti E, et al: Relationship between dose, distance and time in sonic hedgehog mediated regulation of antero-posterior patterning in chick limb. Development 124:4393–4404 (1997).
Zakany J, Kmita M, Duboule D: A dual role for Hox genes in limb anterior-posterior asymmetry. Science 304:1669–1672 (2004).