Experimental approaches for gene regulatory network construction: the chick as a model system

Andrea Streit1,*, Monica Tambalo1, Jingchen Chen1, Timothy Grocott1,$, Maryam Anwar1, Alona Sosinsky2, and Claudio D. Stern3

1Department of Craniofacial Development & Stem Cell Biology, King’s College, London, Guy’s Campus, Tower Wing Floor 27, London SE1 9RT, UK
2Institute of Structural and Molecular Biology, Department of Biological Sciences, Birkbeck College, University of London, Malet Street, London WC1E 7HX, UK
3Department of Cell & Developmental Biology, University College London, Gower Street (Anatomy Building), London WC1E 6BT, UK

Abstract

Setting up the body plan during embryonic development requires the coordinated action of many signals and transcriptional regulators in a precise temporal sequence and spatial pattern. The last decades have seen an explosion of information describing the molecular control of many developmental processes. The next challenge is to integrate this information into logic ‘wiring diagrams’ that visualise gene actions and outputs, have predictive power and point to key control nodes. Here we provide an experimental workflow on how to construct gene regulatory networks using the chick as model system. Keywords: transcription factors, transcriptome analysis, conserved regulatory elements

Introduction

During vertebrate embryonic development the body plan is laid down from a single cell, the fertilised egg. This involves the allocation of multipotent cells to the three germ layers, subdivision of the germ layers into organ primordia, spatial patterning and finally differentiation into special cell types. Thus, multipotent progenitor cells undergo a series of cell fate decisions during which their developmental potential becomes gradually restricted.

Ultimately, the instructions for developmental programmes are encoded in the genome with non-coding regulatory regions and their interacting factors controlling temporal and spatial deployment of cell fate determinants and differentiation genes. While many individual components that govern specific events have been identified, the major challenge is now to integrate this information and to establish predictive models for normal development and disease. Gene regulatory networks (GRNs) are such models: they offer a systems level explanation of developmental processes, organogenesis and cell differentiation (Davidson, 2009, 2010; Levine and Davidson, 2005; Li and Davidson, 2009; Peter and Davidson, 2011a). Their components are transcription factors, which activate or repress downstream target genes by binding to regulatory elements, and the signalling inputs that control their expression. Formation of the body plan requires coordinated and sequential action of many such factors controlling spatiotemporal distribution of cell fate specific proteins and differentiation factors. As cells become specified each population is characterised by a
specific set of transcription factors defining its regulatory state. GRNs establish functional linkages between the signalling inputs, transcription factors and their targets, thus providing a view of cell fate decisions at the molecular level (Fig. 1). In short, GRNs are “wiring diagrams” that explain how cells or organs develop and can highlight ‘inappropriate’ behaviour in disease states. Ultimately, they may also reveal a few critical transcription factors sufficient to impart a specific fate, in a paradigm similar to induced pluripotent stemcells.

GRNs have a hierarchical structure with a clear beginning and terminal states, and therefore have directionality: each state depends on the previous (Davidson, 2006). They define genetic circuits or modules, each with a specific task. It is thus easy to decipher how individual sub-circuits are used repeatedly in different contexts and how the assembly of new modules has allowed cell diversification as well as evolutionary changes. Importantly however, GRNs not only provide information about the genetic hierarchy of network components, but must also identify the cis-regulatory elements that integrate this information. Cis-regulatory analysis is crucial to uncover how individual modules and sub-circuits are deployed and re-assembled within one organism, but also how changes in the regulatory relationships of network components drive evolutionary change, generate diversity and novelty (Davidson, 2011; Hinman and Davidson, 2007; Monteiro, 2012; Peter and Davidson, 2011a).

GRNs are typically depicted as directed diagrams with nodes representing genes and edges representing the connection between nodes and their targets (Fig. 1). Accurate networks provide experimental evidence for the genetic hierarchy as well as for each edge. This requires knowledge of i) the expression of all transcription factors in a specific cell population (defining the regulatory state), ii) the epistatic relationship of these transcription factors generally assessed by functional perturbation experiments and iii) the cis-regulatory elements integrating this information including evidence for direct interaction with appropriate transcription factors. This is a daunting task given the complexity of developmental processes and the genes involved; it is therefore not surprising that to date only few networks fulfil these criteria. A notable exception is the endomesoderm GRN in the sea urchin (Davidson et al., 2002; Oliveri et al., 2002; Peter and Davidson, 2011b; Revilla-i-Domingo et al., 2007; Wahl et al., 2009); see: http://sugp.caltech.edu/endomes/index.html). This network contains a large amount of detail explaining the causal relationship between most components. Thus, it is a good example for the predictive power of GRNs and provides a basis for computational modelling. In Drosophila, similar efforts have led to the discovery of the networks controlling anterior-posterior patterning of the embryo (Nasiadka et al., 2002), mesendoderm development (Wilczynski and Furlong, 2010) and cell fate specification in the eye (Amore and Casares, 2010; Friedrich, 2006; Kumar, 2010) and in ascidians basic GNRs for neural patterning and heart formation have been determined (Imai et al., 2009; Woznica et al., 2012).

In vertebrates, tentative GRNs have been established for cardiac development (Cripps and Olson, 2002), mesendoderm formation (Koide et al., 2005; Loose and Patient, 2004; Morley et al., 2009), dorsal-ventral patterning of the neural tube (Vokes et al., 2007), neural crest (Betancur et al., 2010a; Sauka-Spengler and Bronner-Fraser, 2008) and sensory placode specification (Grocott et al., 2012). However, in most cases the regulatory state of cells at different stages of specification has not been completely defined, many epistatic relationships remain unknown and few cis-regulatory interactions have been verified. This is largely due to the complexity of vertebrate systems, where cell lineage decisions do not follow stereotypic patterns, but are controlled by inductive interactions between large groups of cells combined with cell movements and rearrangements. Therefore, it is difficult to obtain expression data at single cell resolution and to define the regulatory state of whole
cell populations. In addition, many of the vertebrate GRNs integrate data across different species; this is not straightforward due to differences in regulatory interactions that define each species, but also because of differences in experimental approaches, in morphology and speed of development and in some cases difficulties in establishing functional homology of genes (see e.g. (Grocott et al., 2012) for discussion on Dlx genes). Ideally therefore, GRN approaches should focus on a single model organism. This requires a fully sequenced genome, accessibility for gene manipulation in spatially and temporally controlled manner, strategies to assay the expression of many genes in a single sample and in good spatial resolution and finally rapid enhancer analysis to establish direct linkages.

The chick is ideal for this purpose; its genome has been sequenced and is relatively compact. Its phylogenetic position as a non-mammalian amniote is well suited for cross-species sequence comparison e.g. to identify conserved genomic regions. The embryology of the chick is very well described and similar to human development; the embryo is easily accessible for experimental manipulation and because of its relatively slow development, specific cell states can be defined easily. Recent technical advances like transcriptome analysis from small amounts of tissue, efficient knock-down and overexpression strategies, medium throughput transcript quantification and chromatin immunoprecipitation (ChIP) have been adapted to the chick. This makes rapid GRN construction possible without having to resort to cell culture approaches to generate sufficient material. Here, we propose an experimental strategy for GRN construction using the chick as a model (Fig. 2). We focus on strategies for early developmental stages, however with the availability of tissue specific enhancers, of strategies for integration of constructs into the genome permanently and inducible expression systems similar strategies can be applied to later developmental processes. A number of recent reviews discuss the feasibility, advantages and disadvantages of different technical approaches and how they can be applied to particular questions; we refer the reader to these reviews for technical details. The accompanying paper by Khan et al. in this issue provides a complementary computational workflow.

Defining the problem

While GRNs may appear abstract and remote from development, the most essential prerequisite for their construction is a detailed understanding of the biological process under investigation. Only when related to the biological process under investigation do GRNs make sense and are informative. This includes detailed knowledge of fate maps at different stages, cell lineage, inductive interactions that promote and repress certain cell fates and ideally knowledge about fate specification and commitment. This information can only be acquired through careful study of development including the temporal hierarchy of events and tissue interactions. At least for early development, the chick is very appropriate as a system for such studies because of its accessibility for gene manipulation at different times combined with live imaging to study cell behaviour and fate (Kulesa et al., 2010; Rupp and Kulesa, 2007; Stern, 2005a; Voiculescu et al., 2007; Yang et al., 2002). Moreover, its relatively slow development allows the dissection of developmental processes at a resolution not possible in rapidly developing species such as Xenopus and zebrafish as exemplified for neural (Stern, 2005b), neural crest (Betancur et al., 2010a) and placode induction (Grocott et al., 2012; Streit, 2008) as well as neural tube patterning (Balaskas et al., 2012; Ribes and Briscoe, 2009; Vokes et al., 2007), somitogenesis (Pourquié, 2004) and limb development (Towers and Tickle, 2009). Not only is this knowledge essential for GNR construction, it also points to critical steps in the process, important cell fate decision and interactions. To focus experimental strategies and design, this knowledge should be assembled in a flow chart, which will serve as a foundation for the GRN.
Defining the regulatory state

Once the biology has been thoroughly examined the next task is to define the regulatory state of each step in the process. An extensive survey of the literature will provide an excellent resource to define critical molecular markers as well as initial information on epistatic interactions and linkages within the network. Although cross species comparison is very useful, caution should be taken if equivalent cell states cannot be identified, gene expression data are ambiguous and experimental designs vary considerably. Ultimately a close-to-complete knowledge of all transcription factors as well as signals and their effectors is required to assemble a full GRN. With the availability of the chick genome, two strategies have been used successfully over the last few years for unbiased transcriptome analysis: microarrays and to a lesser extent RNA sequencing (RNAseq).

A number of different microarrays are currently available for chick (Antin and Konieczka, 2005) among them a 20K chicken 70-mer oligo array (ARK genomics; modified version from Genomics Research Lab, University of Arizona (Hardy et al., 2011)), an Affymetrix GeneChip covering 28,000 validated and predicted chick genes and an Agilent microarray containing 43,803 probes (Paxton et al., 2010). In addition, several more specific arrays have been designed e.g. for the immune and neuroendocrine systems. The majority of published studies have used Affymetrix arrays (Alev et al., 2010; Bangs et al., 2010; Bento et al., 2011; Buchtova et al., 2010; Cruz et al., 2010; Handrigan et al., 2007; Kimura et al., 2011; Zhang et al., 2010). In general, a fairly large amount of tissue is micro-dissected, RNA isolated using conventional methods and used for probing gene chips. The relatively large size of the chick embryo and ease of dissection make this readily achievable, however it requires precise knowledge of the anatomy and developmental process. More recently, tissue specific promoters driving fluorescent proteins have been used to isolate specific cell populations by FACS sorting, which would otherwise be difficult to dissect (e.g. migrating heart precursors (Bento et al., 2011). In addition, linear amplification protocols in combination with microarray analysis (Chambers and Lumsden, 2008) reduce the amount of tissue required while maintaining reproducibility.

While the use of microarrays has been successful to provide high throughput transcriptome data, this technology also has limitations. These include to a lesser extent lack of comparability across platforms (Liu et al., 2012; Trachtenberg et al., 2012), but importantly the lack of quantitative information with low copy number transcripts often below detection level, and limitations due to the design of the array. In addition, the annotation of many of the chick genes represented in the arrays is fairly poor (Buza et al., 2009) with about 25% of probes still lacking annotations. Therefore, exploitation of the data to their full potential requires a substantial amount of analysis to identify corresponding transcripts reliably by computational tools; in our own experience even then 5–10% of the arrayed oligonucleotide probes on Affymetrix chips fail to identify corresponding genes.

More recently, next generation sequencing RNAseq technology has been developed as an approach for transcriptome analysis. This involves the production of a cDNA library from fragmented Poly-A⁺ RNA, high-throughput sequencing with several uniquely tagged samples being sequenced in a single reaction and mapping of short reads to the reference genome. Although not yet as popular as microarrays, several studies have used this technology for gene discovery and transcriptome profiling in the chick (Li et al., 2012; Wang et al., 2011a; Wang et al., 2011b; Wolf and Bryk, 2011). Unlike microarrays, RNAseq does not require prior knowledge of sequence information; thus it can discover new transcripts. It is more sensitive than arrays and can provide quantitative information; RNAseq is therefore highly suitable to detect low-abundance transcripts. This is important for GRN construction because mRNAs encoding some transcription factors may be present in low amounts. In addition, it informs about the presence or absence of alternatively spliced

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forms and promoter usage. Overall, RNAseq therefore offers a more comprehensive view of the transcriptome in specific cell populations. In chick, the major disadvantage is again the incomplete assembly of the chick genome, which can make it difficult to identify the corresponding genes. However, with a new release now being available (v 4.0; November 2011) and sequence information for different strains being released, RNAseq will become increasingly attractive in this fast moving field. With improved annotation, in the future technologies such as cap-analysis gene expression (Kodzius et al., 2006; Takahashi et al., 2012) will allow not only the identification of the transcriptome, but also of all genes actively transcribed in a specific cell population by mapping the transcription start sites within promoters. This will be a major advance in network construction and analysis.

The above approaches will generate a comprehensive gene list that defines the regulatory state of specific cell populations, i.e. the sum of all regulatory genes and their targets; this essentially provides the toolkit for network building. This knowledge is required for different time points during cell fate specification to give a dynamic view of development. However, as with all molecular screens, the design is critical to return transcripts relevant to the process, while excluding others that may play more general roles.

While temporal information on gene expression is rapidly generated either by the methods described above or by qPCR, the acquisition of spatial expression data is more laborious, but absolutely required for successful GRN establishment. The expression of all network components should be validated by in situ hybridisation. In addition, online resources like Geisha (Gallus Expression in Situ Hybridisation Analysis http://geisha.arizona.edu/geisha) and the echickatlas (http://www.echickatlas.org/ecap/home.html) provide useful resources for expression data. This analysis will distinguish genes with ubiquitous, broad, mosaic or cell population-specific expression and thus be invaluable to define the regulatory state. For example, although the presence of ubiquitous transcripts may be important, they may or may not provide any information that is specific for the developmental process. Together with temporal profiles, spatial resolution allows prediction of a genetic hierarchy and the assembly of a preliminary network as a testable model.

Establishing a hierarchy: perturbation experiments and analysis of network components

While the precise knowledge of gene expression is critical for building a GRN, the regulatory interactions between network components must be determined by functional perturbation experiments. The aim of these experiments is to establish whether changes in the endogenous level of transcripts (loss- or gain-of-function) results in repression or activation of downstream targets. While targeted mutagenesis in chick is not established, in the last 10–15 years transient transgenesis has become a routine technique. Importantly, the young chick embryo lends itself to temporally and spatially controlled knock-down and misexpression, thus allowing the perturbation of gene function at the appropriate time and in the tissue relevant to the process under investigation (see Fig. 3). This circumvents problems when genes have multiple functions at various time points in development. Below we will briefly consider the many strategies available in the chick to perform such experiments in vivo and in explants in culture (Tab. 1). Although we focus on early events, with the availability of inducible constructs, of methods for transgene integration into the genome and tissue specific enhancers similar strategies can be used to examine later processes. Many of the approaches described can be performed in the presence or absence of translation inhibitors to determine direct targets of a signal or transcription factor.

Although transfection (Albazerchi et al., 2007; Geetha-Loganathan et al., 2011), sonoporation (Ohta et al., 2008) and retroviruses (Hou et al., 2011) have been used for transgenesis, electroporation is by far the most widespread method in early embryos used for transient misexpression and knock-down approaches in chick (Hatakeyama and Shimamura,
For genesilencing, electroporation of small interfering RNAs and modified antisense oligonucleotides (morpholinos) are widely used, while dominant negative constructs interfering with endogenous protein function are useful tools, especially for probing the function of transcription factors and receptors.

**Loss of function approaches**—Double stranded RNAs (dsRNA) targeting the gene of interest provide a powerful gene silencing approach. Recently a number of vectors have been designed that generate small interfering RNAs of 20–21 nucleotides, short hairpin RNAs or pre-miRNAs, which are processed into small dsRNAs by the cellular machinery to lead to sequence-specific target mRNA degradation (Bron et al., 2004; Das et al., 2006; Hu et al., 2002; Katabira and Nakamura, 2003); for review: (Sauka-Spengler and Barembaum, 2008; Hou et al., 2011). Some of these vectors also contain fluorescent proteins allowing easy detection of cells carrying the transgene. The main advantage of this strategy as compared to morpholinos is the unlimited supply of plasmid and importantly that gene knock-down can be verified by in situ hybridisation. However, non-specific effects including activation and loss of unrelated transcripts have been reported in particular in young chick embryos (Mende et al., 2008) demonstrating the critical importance of appropriate controls (see below).

Antisense morpholinos provide a good alternative, especially for early embryos, and result in reproducible and reliable gene inactivation (Basch et al., 2006; Christophorou et al., 2010; Kos et al., 2003; Mende et al., 2008; Sheng et al., 2003; Strobl-Mazzulla et al., 2010; Voiculescu et al., 2008). Antisense morpholinos target either the translation start site to interfere with the initiation complex or splice junctions resulting in exon deletion or intron inclusion. If appropriately designed, the latter generate truncated proteins by introducing premature stop codons. For translation blocking morpholinos, knock-down efficiency must be determined by antibody staining, which may be difficult as often specific antibodies are not available. In case of splice-blocking morpholinos the efficiency is assessed by RT-PCR. In addition, we have recently adapted morpholino-mediated knock-down for tissue explants using the Endoporter system (GeneTools) for delivery; like in vivo this strategy generates efficient and reliable knock-down in particular as the tissue can be cultured in the presence of morpholinos.

Both dsRNA and morpholino approaches require careful controls for off-target, non-specific effects, for knock-down specificity and in case of morpholinos, for toxicity. Standard control morpholinos serve as general controls (toxicity, electroporation), while 6 base pair mismatched morpholinos or dsRNAs should control for off-target effects. Ideally, two different antisense oligonucleotides with distinct target sites should be used as well as appropriate rescue experiments by co-electroporation of expression constructs that lack the target sequence or by downstream targets. Thus, each knock-down strategy must be carefully controlled before it can be used to determine the epistatic relationship among the components of gene regulatory networks. Once this is achieved, electroporated tissues can be collected to assess the changes in endogenous levels of network components using different strategies.

**Dominant-negative constructs, constitutive repressor and activator forms**—The use of inhibitory or constitutively active forms of proteins, or fusion proteins that generate constitutive active or repressing transcription factors may also be informative to determine the genetic hierarchy within a gene network. Dominant negative constructs generate mutated proteins, which compete with endogenous proteins expressed in the same cell, while constitutively active proteins generally contain mutations that mimic their active state (e.g. phosphorylation). This strategy has been widely used to interfere e.g. with signalling pathways by constructing receptors that lack intracellular domains or are active in
the absence of ligand, or by providing constitutively active downstream mediators (Bobak et al., 2009; Linker and Stern, 2004; Suzuki-Hirano et al., 2005; Suzuki et al., 1994; Timmer et al., 2002), or to interfere with transcription factor function by deleting either the DNA binding or trans-activating domains, but leaving protein interaction domains intact (Cossais et al., 2010; Rallis et al., 2003). In addition, transcription factors are turned into constitutive repressors or activators by fusing their DNA binding domain to repressors domains like the engrailed repressor or activator domains like VP16 or E1A (Bel-Vialar et al., 2002; Christophorou et al., 2009; Glavic et al., 2002; Hollenberg et al., 1993; Horb and Thomesen, 1999; Kolm and Sive, 1995; Li et al., 2009; Steventon et al., 2012; Wheeler and Liu, 2012). The latter are particularly useful for transcription factors that recruit either co-repressors or co-activators depending on the cellular context. In this case, comparison to misexpression of wild type forms can determine whether changes of downstream targets are due to repression or activation by the factor under investigation. These approaches have been very successful in other organisms, but have recently also been used in the chick. As for knockdown approaches, careful controls must be designed to ensure specificity and test for off-target effects. In particular, since inhibitory constructs act by competition with endogenous proteins, they largely depend on overexpression at non-endogenous levels. This frequently leads to mislocalisation within the cell (e.g. signalling second messengers, transcription factors and co-factors whose nuclear localisation is normally tightly controlled may become constitutively nuclear when overexpressed), or participation in non-specific protein-protein interactions (e.g. constitutively active and dominant negative receptors may bind non-specifically to co-receptors), which in turn may trigger unintended and off-target perturbations.

**Gain-of-function approaches**—Mis- or overexpression strategies are well established in the chick. As discussed above electroporation is the method of choice to generate transient expression of DNA constructs and assess their effect on network components. Different vectors are currently available; for ubiquitous transgene expression most vectors use a CMV immediate early enhancer and the chick β-actin promoter and some contain an internal ribosomal entry site followedby GFP or RFP to allow easy tracking of electroporated cells (Ishii and Mikawa, 2005; Itasaki et al., 1999; Nakamura et al., 2004; Odani et al., 2008; Voiculescu et al., 2008). More recently, new vectors were developed using Tol2 transposon-mediated gene transfer for stable integration into the genome; combined with tetracycline-inducible expression and inducible or tissue specific Cre/loxP systems misexpression can be achieved in a tissue-specific manner at the desired time (Sato et al., 2007; Takahashi et al., 2008; Yokota et al., 2011). Together, these approaches are powerful tools to perform gain-of-function experiments in a wide variety of tissues in a temporally and spatially controlled manner.

**Measuring changes in gene expression**—One of the most critical steps to elucidate the network architecture is to monitor changes in gene expression after perturbation experiments. Although qualitative methods like in situ hybridisation may provide important, often critical information (especially for when complex spatial changes of expression occur), they are unlikely to detect subtle changes and are impractical for large networks with many components. Therefore, quantitative or semi-quantitative methods are required, which ideally allow measuring many transcripts in the same sample.

With the development of sensitive methods it has become possible to use small tissue samples like single embryos, electroporated tissue dissected from embryos or few explants for quantitative analysis of gene expression (Strobl-Mazzulla et al., 2010; Taneyhill and Bronner-Fraser, 2005). To evaluate the effects of gene knock down or overexpression, samples from control and experimental tissues are compared. Currently two main
approaches have been used to determine mRNA levels for network analysis, quantitative real-time PCR (qPCR) and NanoString nCounter.

qPCR is widely used providing a reliable, sensitive and kinetic approach to evaluate changes of gene expression after experimental perturbation. After cDNA synthesis, qPCR uses fluorescent dyes or probes that bind double stranded DNA to quantify the increase in specific PCR products during the exponential phase of amplification. Internal standards serve as controls for data normalisation and the ratio of control to experimental values determines the fold change. Thus, qPCR detects repression and activation of network components after experimental perturbation. While qPCR is a powerful method to quantify transcripts, it is laborious if a large number of network components are to be analysed.

NanoString nCounter alleviates this problem providing a multiplex strategy for sensitive quantification of up to 500 transcripts in a single sample (Geiss et al., 2008; Malkov et al., 2009). NanoString is a hybridisation based technique that uses two target specific short oligonucleotide probes: one capture probe to bind the hybridised RNA to a solid surface and a reporter probe tagged with a unique barcode of fluorescent dyes. The combinatorial use of different fluorescent tags allows the detection of large numbers of transcripts in a sample. Captured samples are analysed by a fluorescent microscope, which counts the number of times each bar code is detected. Standard probe sets contain positive and negative controls, as well as housekeeping genes for normalisation of the data in addition to all regulatory genes that characterise the network. Like qPCR, NanoString provides quantitative information about the changes in gene expression after perturbation by comparing control and experimental conditions. Both approaches generate highly reproducible results with equal fidelity (Materna et al., 2010). However, NanoString has the advantage that a large number of transcripts can be analysed simultaneously thus simplifying the analysis of complex networks considerably. In the future, with continuously improving methods and decreasing prices, RNAseq may become the method of choice to analyse perturbation experiments.

**Enhancer discovery and validation of predicted interactions**

For a complete GRN that models specific processes during development ideally, each edge within a network will require experimental validation. Although perturbation analysis provides critical information about the network architecture it cannot distinguish which of the interactions are direct. Ultimately, this requires the identification of cis-regulatory elements that integrate transcriptional inputs as well as their interacting transcription factors. In the first instance, perturbation experiments will point to important nodes in the network and thus highlight candidates for which such cis-regulatory analysis is high priority. In addition, different clustering algorithms such as hierarchical, K-means and self organising maps (Johnson, 1967; Kohonen, 1990; MacQueen, 1967) can be used on multiple datasets (e.g. NanoString data) to identify small groups of co-regulated genes, which may share some transcriptional input. Computational methods and sequence alignment must then be employed to predict putative enhancer regions for individual genes, but also to discover shared motifs among co-regulated transcripts (see Khan et al., 2012).

One major challenge is to predict the most likely cis-regulatory modules (CRMs) that control gene expression in the tissue or cells of interest; a workflow to achieve this is described by Khan and colleagues in this issue (Khan et al., 2012). This is critical since currently the major bottleneck to verify connections is the slow process of validating CRM activity in vivo including analysis and experimental verification of interacting transcription factors. This seems like an impossible task, however in recent years, the chick has proven to be an excellent system to perform such analysis rapidly as illustrated by the pioneering work of the Kondoh group (Inoue et al., 2007; Kondoh and Uchikawa, 2008; Matsumata et al.,

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Appropriate reporter vectors have been designed that allow rapid cloning of putative CRMs to drive the expression of fluorescent proteins like YFP, GFP, RFP, cherry or cerulian. These constructs are electroporated into large regions of the chick embryo together with an ubiquitously expressed fluorescent protein to control for electroporation efficiency and specificity of the putative CRM (Fig. 4). This strategy allows the evaluation of a relatively large number of enhancers at reasonable cost and in a relatively short time. This strategy has been very successful in recent years to identify active enhancers that control gene expression in specific tissues (Barembaum and Bronner-Fraser, 2010; Betancur et al., 2010b; Betancur et al., 2011; Iwafuchi-Doi et al., 2011; Neves et al., 2012; Prasad and Paulson, 2011; Sato et al., 2012; Sato et al., 2010; Strobl-Mazzulla et al., 2010). However, in the future the development of multiplex strategies for CRM validation will greatly speed up this process. Currently, a multiplex approach is used in the sea urchin that allows testing of 100 CRMs in a single experiment (Nam and Davidson, 2012), however this strategy is not directly applicable to the chick system.

Once identified, active CRMs are analysed computationally for the presence of transcription factor binding sites (Khan et al., 2012). While this analysis is likely to return a large number of possible binding factors or simply families of transcription factors, good candidates are those genes identified by microarrays or RNAseq (see above) to be present in the tissue of interest. Subsequent experiments will need to verify the interaction of such candidates with the CRM and whether this interaction is necessary for CRM activity. Traditionally, electromobility shift assays have been used to show physical protein-DNA interactions in vitro. However, more recently chromatin immunoprecipitation for small tissue samples (micro-ChIP), coupled with qPCR, has been used successfully to confirm transcription factor binding to specific enhancer regions in vivo. Finally, mutagenesis of transcription factor binding sites within the CRM as well as knock down of the appropriate transcription factor are critical to demonstrate the requirement of the interaction for CRM function.

While the above strategy is likely to identify active CRMs, the question remains of whether these are truly the active enhancers that control expression of the gene of interest in vivo. Ultimately, this issue needs to be resolved using transgenic approaches in other species where the CRM is modified or deleted from its normal location in the genome.

**Putting it all together: assembling a network**

As discussed above temporal and spatial analysis of network components allows the assembly of a preliminary network as a working model. The perturbation experiments combined with quantification of the expression of all network components will establish a genetic hierarchy, define upstream regulators and targets and point to key nodes within the network. In short, the approaches will reveal information about the network architecture. However, establishing the GRN is an iterative process with each iteration refining the network further. Ideally, experimental manipulations should be performed as time course experiments and in the presence of translation inhibitors, which will indicate dynamic changes and help to distinguish direct from indirect interactions. However, only the combination of perturbation experiments with identification of cis-regulatory elements that control gene expression and their interacting transcription factors will provide the necessary evidence.

One of the key challenges however, is to organise the data generated and to incorporate them into a logical network. The available networks for sea urchin endo- and mesoderm specification provide an excellent example for data organisation in interaction tables combined with gene expression data in the embryo (Davidson et al., 2002; Peter and Davidson, 2011b) see: http://sugp.caltech.edu/endoomes/index.html. In addition, computational tools are indispensible for network assembly. Our favourite programme is
BioTapestry (http://www.biotapestry.org/), because it allows easy navigation between different regions (regulatory states) and times, as well as the ability to follow genetic interactions easily. BioTapestry was specifically designed for GNR visualisation, but also integrates perturbation data, suggests alternative models and helps with data interpretation by pointing to critical interactions to be tested (Longabaugh et al., 2005, 2009). Programmes like BioTapestry allow the organisation of complex data sets into logical circuits and are thus essential for network construction.

In addition, new computational approaches for inferring GRNs are emerging continuously. These approaches allow the reconstruction of networks from complex high-throughput data and are powerful tools to predict hubs and interactions within a network, which can then be tested experimentally (Basso et al., 2005; Hartemink, 2005; Werhli et al., 2006).

**Conclusion**

The chick has been an embryological model system for hundreds of years and thus provides a wealth of information. Many developmental processes have been described in detail and particularly its slow development has made it possible to dissect their timing. Combined with the ease of experimental manipulation, the availability of the chick genome, advances in adapting molecular methods and medium- to high-throughput gene expression analysis and the design of new vectors, have now made the chick a most attractive model to construct gene regulatory networks. In depth understanding of the biology remains key to design appropriate molecular screens, identify network components and their epistatic relationship. Computational methods for CRM and insulator prediction, data analysis and network inference complement the experimental approaches and together provide a powerful strategy for network construction.

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Figure 1.
An example of a simple gene regulatory network. a. In this network, signal 1 from tissue A to signals to tissue B via a receptor (≫); this triggers the expression of transcription factors 1 and 2 (gene 2 and 3) and several downstream targets are activated (genes 4–6). These in turn regulate each others’ expression as indicated by the coloured arrows. Positive interactions are depicted as arrows, negative interactions as bars. At a later time, tissue C emits a second signal, which leads to changes in gene expression in cells nearby, while cells far away are not exposed to signal 2. This leads to differential gene expression in tissue B1 and B2 depending on the transcription factors interactions and signalling input. b. The initial stage in network construction is defining the regulatory state: the sum of genes expressed in each tissue at different times and the signals received from neighbouring tissues (see Fig. 2 stage...
2). c. Perturbation experiments suggest interactions and hierarchy (see Fig. 2 stage 3). d. Confirmed interactions after enhancer discovery and testing (Fig. 2 final stage).
Figure 2.
Experimental workflow for building a gene regulatory network. Details for each step are described in the text. Generating a GRN is an iterative process, in which each perturbation experiment informs about the network architecture; integration of new information into the network points to novel hypotheses that can then be tested experimentally. Bioinformatics approaches are required to predict regulatory interactions and conserved regulatory modules (CRMs). In vivo testing of CRM activity and their regulation feeds back to the network.
Figure 3.
Gain- and loss-of-function experiments in chick embryos. a: Exogenous DNA or oligonucleotides are transfected into chick embryos by electroporation. b: eGFP was electroporated into the ectoderm of a primitive streak stage embryo. GFP fluorescence can first be detected about 3–4 hours after electroporation. After overnight culture the neural plate, the non-neural and extraembryonic ectoderm carries the GFP construct. c and d: electroporation of a Pax2-specific morpholino (MO; green) (Mende et al., 2008) at primitive streak stages into otic precursors leads to loss of Pax2 protein (red) in electroporated cells. c and d show the left and right side of the same embryo, respectively. Note: only few cells carry Pax2 MO on the left hand side, while most are electroporated on the left hand side; this leads to a change in placode morphology (Christophorou et al., 2010).
Figure 4.
Testing enhancer activity in chick embryos.
a. Diagram showing the GFP-reporter construct containing the putative enhancer, a minimal TK promoter and eGFP; RPF is driven by chick β-actin and CMV promoter. Embryos are electroporated at primitive streak stages and cultured until they have reached the stage when enhancer activity is expected. b–e. The embryo was electroporated at primitive streak stages with ubiquitous RFP and GFP driven by an otic Eya1 enhancer (Ishihara et al., 2008). After overnight culture the embryo has reached the 13-somite stage and shows enhancer activity in the otic placode b. bright field image. c. RFP expression is widespread. d. GFP is specifically expressed in the otic placode. e. Overlay of bright field and GFP image. White circles indicate the otic placode. mb: midbrain; hb: hindbrain, ov: optic vesicle; som: somite.
### Table 1

Summary of perturbation experiments as described in the text

<table>
<thead>
<tr>
<th>Perturbation Method</th>
<th>MOs/Small interfering RNA (siRNA)</th>
<th>Misexpression</th>
<th>Dominant negative forms of TFs, receptors</th>
<th>Constitutive activator or repressor forms of TFs</th>
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<tr>
<td>Morpholino</td>
<td>MOs complementary to the transcription start site block translation; MOs complementary to splice junctions result in exon deletion or intron inclusion</td>
<td>Ectopic expression of the gene of interest by electroporation or transfection; grafting beads or transfected cells for signalling molecules</td>
<td>Expression of genes that lack a critical domain (e.g. intracellular domain of receptor, co-factor binding domain of TF) and interferes with endogenous proteins</td>
<td>Expression of TF-fusion constructs where the DNA-binding domain is fused to a constitutive repressor (e.g. EnR) or activator (VP16, E1A)</td>
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<td>Small interfering RNA (siRNA)</td>
<td>RNA degradation</td>
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Basch et al., 2006; Christophorou et al., 2010; Kos et al., 2003; Mende et al., 2008; Sheng et al., 2003; Strobl-Mazzulla et al., 2010; Voiculescu et al., 2008

Bron et al., 2004; Das et al., 2006; Hu et al., 2002; Katahira and Nakamura, 2003

Nakamura, et al., 2004

Bobak et al., 2009; Linker and Stern, 2004; Suzuki-Hirano et al., 2005; Suzuki et al., 1994; Timmer et al., 2002; Cossais et al., 2010; Rallis et al., 2003

Bel-Vialar et al., 2002; Christophorou et al., 2009; Glavic et al., 2002; Hollenberg et al., 1993; Horb and Thomsen, 1999; Kolm and Sive, 1995; Li et al., 2009; Steventon et al., 2012; Wheeler and Liu, 2012

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