Gallus Expression In Situ Hybridization Analysis: A Chicken Embryo Gene Expression Database[1]

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ABSTRACT With sequencing of the chicken genome largely completed, significant effort is focusing on gene annotation, including acquiring information about the patterns of gene expression. The chicken embryo is ideally suited to provide detailed temporal and spatial expression information through in situ hybridization gene expression analysis in vivo. We have developed the Gallus expression in situ hybridization analysis (GEISHA) database and user interface (http://geisha.arizona.edu) to serve as a centralized repository of in situ hybridization photos and metadata from chicken embryos. This report describes the design and implementation the GEISHA database and Web site and illustrates its usefulness for researchers in the biomedical and poultry science communities. Results from a recent comprehensive expression analysis of microRNA expression in chicken embryos are also presented.

Key words: chicken, database, in situ hybridization

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INTRODUCTION

The chicken has been a valuable model for experimental biology for more than 100 yr and is unique among biomedical model organisms because it is also an agriculturally important species. For the agricultural and biomedical research communities, an important research focus has been the understanding of gene function, which for many genes remains unknown.

An important prerequisite to understanding gene function during embryo development is the mapping of gene expression patterns. In this regard, the chicken embryo is ideally suited for in situ hybridization expression analysis. As an amniote, avian embryo development is remarkably similar to mammals, including humans, and chicken embryos can be obtained in large numbers at low cost. In situ hybridization protocols for avian embryos have also been carefully optimized (Nieto et al., 1996; Bell et al., 2004; Darnell et al., 2006). For these reasons, considerable effort has been directed toward visualizing gene expression patterns during chicken embryogenesis. To date the temporal and spatial expression patterns for only a fraction of genes are known, although a large amount of data resides in the published literature and many laboratories have accumulated unpublished in situ hybridization expression data. As biological investigation moves from understanding individual gene function to the interrelated function of gene networks, research efforts have been hindered by the inability to easily retrieve and query expression patterns for groups of genes for which information is scattered across the literature, unpublished, or not yet determined.

We have developed the Gallus expression in situ hybridization analysis (**GEISHA**) database and user interface (http://geisha.arizona.edu) to serve as a centralized repository of in situ hybridization information in chicken embryos (Bell et al., 2004). The goals of the GEISHA project are to use high throughput in situ hybridization analysis to map expression of all differentially expressed genes in the chicken embryo, to acquire additional reliable expression data from other researchers and from the published literature, and to present this information through a Web-based user interface. Availability of an increasingly accurate chicken genomic sequence has enabled integration of probe sequences and expression data with the genome. This report describes implementation and capabilities of the GEISHA database, plans for new tools, and a recent targeted high throughput screen using novel locked nucleic acid (**LNA**) probes to examine the expression of 135 microRNA (**miRNA**) genes.

THE GEISHA DATABASE

The GEISHA database consists of a Web-based laboratory information management database for managing im-

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ages and associated metadata, and a Web site that enables users to search and display images and related gene annotation. It is written in Java and uses open source software including Apache Tomcat and Postgresql. The GEISHA database interfaces with the Ensembl genomic database to link GEISHA entries to genes via genomic location. Future integration is anticipated with the University of California, Santa Cruz, and National Center for Biotecholgoy Information chicken genome browsers.

In situ hybridization expression information housed in the GEISHA database is derived from situ hybridization screens performed in our laboratory, unpublished data from other laboratories, and expression information from the published literature. For in-house screens, cDNA for probe templates have been obtained from several embryonic cDNA libraries, and we have also performed targeted screens to map expression of specific classes of genes. We have recently mapped expression of most known chicken microRNA (Ason et al., 2006; Darnell et al., 2006) and are presently screening all known transcription factors using cDNA identified in the Biotechnology and Biological Sciences Research Council Chick EST database (Boardman et al., 2002). A large amount of unpublished expression data also resides in laboratories around the world, and a submission page is being developed for off-site incorporation of data into GEISHA. For unpublished expression data, a key concern is probe identity, and all acquired information will be carefully curated prior to inclusion in the GEISHA database. Finally, agreements have been reached with most relevant journals (*Anatomical Record, Developmental Biology, Developmental Dynamics, Gene Expression Patterns, International Journal of Developmental Biology, Mechanisms of Development, and Proceedings of the National Academy of Sciences of the United States of America*) to display expression information on the GEISHA database. Incorporation of published data represents a long-term curation effort.

All expression information is organized according to the mapped location of probe sequences on the chicken genome. The cDNA sequences used for probe generation are mapped to the genome using the Ensembl Exonerate or SSAHA search tools (Ning et al., 2001; Slater and Birney, 2005). Because expression information from a single gene may be obtained using probes generated from more than 1 cDNA template and because probes for a single gene may have different sequences due to alternative splicing, each probe and corresponding in situ hybridization images are stored and displayed together. Expression information for all probes mapping to an individual gene are displayed on one gene expression report page (Figure 1). Each probe and its corresponding genomic information are displayed together, with images organized as thumbnails according to stage. For each probe, the gene name, description, links to its genomic location on the Ensembl database, a graphical representation of the probe mapped onto the local genomic region, and the DNA sequence of the probe template are displayed. If in situ hybridization data derives from a publication, a direct link to the PubMed entry is provided along with the citation and copyright attribution.

Although presently a comprehensive chicken anatomical ontology is not available, we have generated an abbreviated anatomical atlas based on the mouse atlas, with appropriate species-specific changes. All images have been annotated according to the anatomical location of their expression patterns, and this information is shown to the right of the images in each row on the gene expression report page. Clicking on an anatomical term highlights the relevant images. Clicking on an individual thumbnail loads a higher resolution image, along with figure legend information if the image was obtained from a publication.

Information in the GEISHA database is accessed through several browse and search tools on the GEISHA homepage (Figure 2). Gene name or gene description, anatomical location, embryo stage, GEISHA ID, publication author, and Ensembl Gene ID can be searched using word entries and the search window at upper right. Search returns appear as tables of images or lists of gene entries. Expression information can also be retrieved using the browse functions on the left sidebar according to anatomical location, embryo stage, gene name, GEISHA ID, or using combinations of these parameters. Accessing "anatomical location" on the sidebar returns a page showing a hierarchical listing of anatomical terms used to classify expression patterns. Some subheadings are expandable to show additional anatomical terms. Click boxes allow the user to define individual or various combinations of anatomical terms for searching. Accessing "browse by stage" returns a page showing representative thumbnails images for each stage between HH stages 1-27. Clicking on one of the representative-stage images returns a table showing thumbnails of all images in the GEISHA database of that stage. Mouse-over of each image in the table displays a pop-up window showing the gene name or GEISHA ID corresponding to the probe used to obtain the expression pattern. Clicking on an individual image returns the corresponding Gene Expression Report page. Accessing "search by gene name" allows users to choose from an alphabetical list of official gene names and any alternative names that may be recognized in the literature. A list of GEISHA ID is also available. Finally, the multiple parameter search option permits entry retrieval using any combination of embryo stage, anatomical location, gene name or description, GEISHA ID, and publication author.

miRNA EXPRESSION IN CHICKEN EMBRYOS

As indicated above, the high throughput in situ hybridization laboratory of the GEISHA project provides the resources to investigate the expression patterns of entire classes of RNA sequences that are of particular interest to researchers. In addition to an ongoing screen of all known transcription factor genes in the chicken genome, we have recently completed a comprehensive screen of

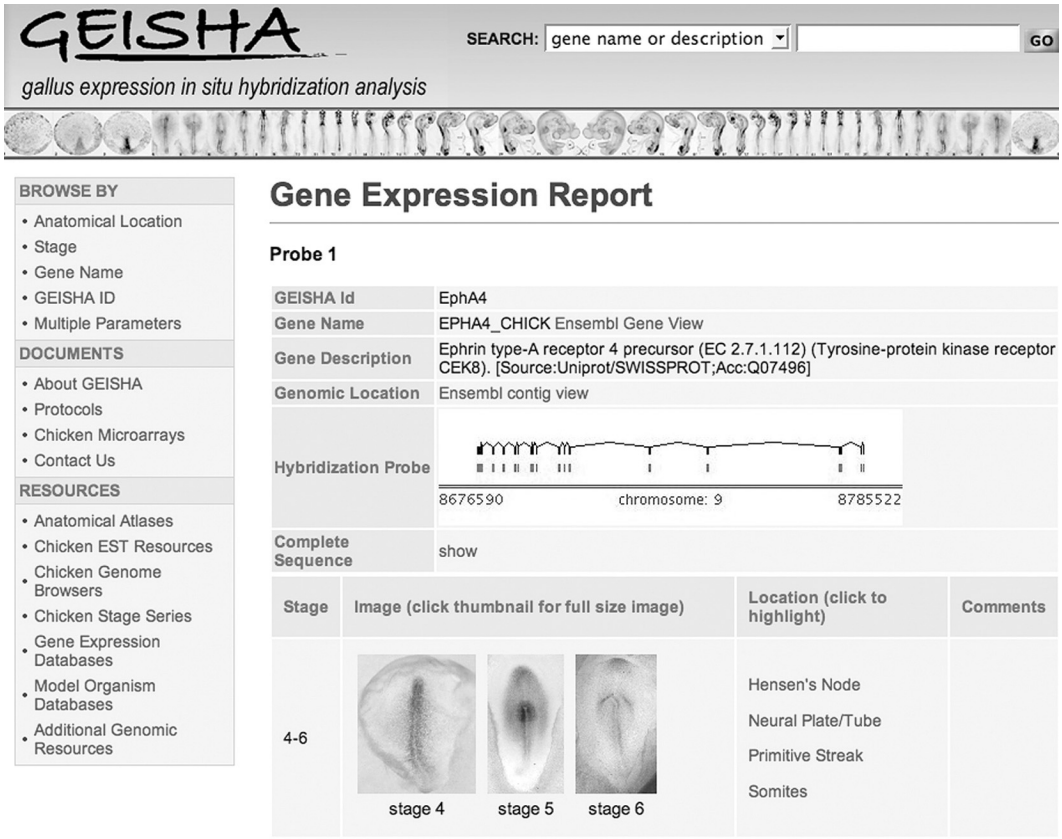

Figure 1. Screen capture showing the layout for a typical gene expression report page, in this case for the receptor tyrosine kinase EphA4. Information includes the official gene name, the gene description from the Ensembl genome browser, plus links to the corresponding Ensembl GeneView and ContigView pages. A schematic shows the location of probe sequence (red) relative to the entire gene (black). Images are presented in thumbnail format in stage order, with the anatomical localization of expression noted in the location column to the right of the images.

miRNA expression through d 4 of embryogenesis. The miRNA are small noncoding RNA that can regulate protein abundance by binding to target sequences in 3′ UTR and inhibiting mRNA translation. The miRNA regulate many important processes, including cell fate decisions, morphogenesis organogenesis, metabolism, and cell proliferation and survival (Ambros, 1989; Reinhart et al., 2000; Brennecke et al., 2003; Dostie et al., 2003; Calin et al., 2004; Alvarez-Garcia and Miska, 2005; Giraldez et al., 2005). The miRNA function has also been implicated in regulating stem cell renewal and the onset of certain cancers (Alvarez-Garcia and Miska, 2005; Croce and Calin, 2005; Hatfield et al., 2005; Hammond, 2006).

Because mature miRNA are just 21-22 nt in length, standard in situ hybridization protocols using antisense RNA probes are not useful. Recently, a novel type of DNA analogue called an LNA has been developed that exhibits superior hybridization kinetics and enhanced biostability, dramatically enhanced affinity toward DNA and RNA, and superior discrimination between matched and mismatched target sequences (Koshkin et al., 1998; Mctigue et al., 2004). The 20-25 nt LNA hybridize to target RNA sequences in vivo with extremely high specificity and hybrid stability and have proven effective for visualizing miRNA in embryos (Koshkin et al., 1998; Weinholds et al., 2005; Kloosterman et al., 2006). To investigate miRNA expression during chick embryo development, LNA-containing oligonucleotides antisense to 111 distinct chicken miRNA sequences transcribed from 135 miRNA genes were used for in situ hybridization analysis. Seventy-five mRNA exhibited ubiquitous or differential expression in embryos between 15 and 52 h of incubation (HH stages 2-25). Whereas relatively few miRNA were detected during gastrulation, expression of many miRNA became detectable during formation of the major organ systems (Figure 3). The miRNA were detected in the heart and skeletal muscles, the forming gut, and in the ectoderm, limb buds, and blood vessels. The miR-1 showed strong expression in the myocardium and in skeletal muscles cells of the somites (Figure 3A, 3B), whereas miR-206 was detected in somitic muscle cells but not in the heart (Figure 3C). The miR-126 was first detected in the earliest endothelial cells at stages 7-8 and was later expressed in all blood vessel endothelial cells (Figure 3D). More than 30 miRNA were detected in defined regions and cell layers of the central nervous system, including miR-9, which labeled subsets of cells in all brain vesicles and in the spinal cord (Figure 3E). Additional representative central nervous system specific expression patterns are shown in Figures 1F-H (miR-100 and -124).

A large cohort of miRNA showed generally ubiquitous expression in 1 or more germ layer-derived tissues, for

Figure 2. Screen capture of the Gallus expression in situ hybridization analysis (GEISHA) homepage. Searches can be performed using the search box at upper right, whereas browse functions are accessible in the left sidebar.

example miR-106, which was detected in most mesodermal cells at stage 14 (Figure 3I), whereas others were detected only in a subset of mesoderm (e.g., miR-140 in notochord, Figure 3J; and miR-144 in blood islands, Figure 3K). The miR-205 was detected broadly throughout the ectoderm and at higher levels in the pharyngeal arch and limb bud ectoderm (Figure 3L). Twenty additional miRNA were detected in limb buds (data not shown), suggesting important multiple functions in limb development. In a few cases, expression was apparently limited to a single cell type. For example miR-375, which has been shown to play a role in regulating insulin secretion (Poy et al., 2004), was detected in a few cells in the pancreas rudiment from stage 12 onward (Figure 3M, 3N). A more comprehensive description of miRNA expression in chick embryos can be found in Darnell et al. (2006) and by visiting the GEISHA Web site (http://geisha.arizona.edu).

SUMMARY

The objectives of the GEISHA project are to house and display chicken whole mount in situ hybridization images, annotation, and related genomic information. GEISHA consists of a Web-based database for managing images and associated metadata, and a Web site user interface that enables users to browse, search, and display images and related gene annotation. Information is acquired from high-throughput expression screens, literature curation, and unpublished information from other laboratories. We recently redesigned the database to incorporate more complex metadata and enhanced search functions, including a more comprehensive anatomy. Information is accessed through browse and search functions, and additional search capabilities are planned, including searching by gene ontology terms, which classify all proteins according to molecular function, cellular component, and biological process. More sophisticated sequence mining capabilities are also under development that would allow users to retrieve genomic sequence information from groups of genes showing expression patterns of interest. Large-scale screening efforts encompassing entire classes of mRNA have been completed or are underway, including all identified miRNA and transcription factors. As the volume of expression information housed in the database increases, GEISHA will become an increasingly valuable resource for the chicken biomedical and agricultural research communities.

METHODS

Embryo Collection and Preparation

Fertile chicken eggs (HyLine, Spencer, IA; not a commercially available source) were incubated in a forced-draft, humidified incubator at 37.5°C for 0.5 to 5 d, depending on the stages desired. Embryos were collected into chilled chick saline (123 m*M* NaCl in nanopure wa-

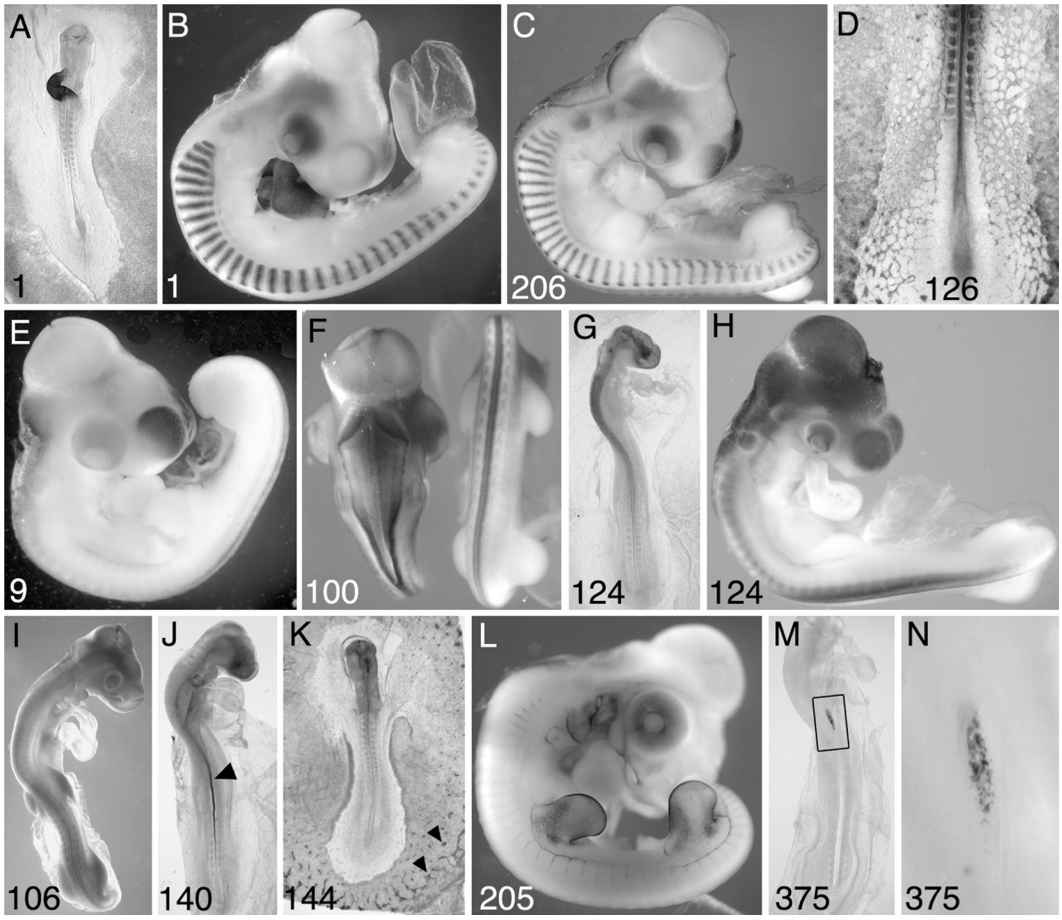

Figure 3. Representative images of microRNA (miRNA) expression patterns in chicken embryos. miR-1 is detected in the myocardium of the heart at stage 12 (A), and in the myocardium and myotomal skeletal muscle cells at stage 22 (B). miR-206 is expressed in skeletal muscle cells of the myotome but is not detected in the myocardium (C). miR-126 is expressed exclusively in blood vessel endothelial cells (D). (E-H) Representative miRNA that localize to the central nervous system. I) miR-106 is detected in most mesodermal derivatives, whereas miR-140 is detected in the notochord (J) and mir-144 in the blood islands (K). miR-205 is expressed almost exclusively in the ectoderm (L). (M, N) miR-375 expression in the developing pancreas.

ter), removed from the vitelline membrane and cleaned of yolk. Extraembryonic membranes and large body cavities (brain vesicles, atria, allantois, eye) were opened to minimize trapping of the in situ reagents. Embryos were fixed in fresh, cold 4% paraformaldehyde in PBS overnight at 4°C. Embryos were maintained at or near 4°C during collection because significant loss of labeling was correlated with increased time at room temperature during collection, especially for miRNA.

Embryos were rinsed in PBS, then in PBS plus 1% Triton X-100 (PBT) for mRNA, and dehydrated by steps (25, 50, 75, 100, 100%) into methanol before being cooled to –20°C overnight (or up to 10 d). For micro RNA, an equal concentration of Tween-20 was substituted for the Triton in this and all subsequent steps in which Triton is normally used. Rehydration reversed this series. Embryos were rinsed 2× in PBS, and older embryos were treated with proteinase K: stages 8-13 and 14-18 at 10 μg/mL of proteinase K for 10 and 20 min, respectively; stages 19 and older at 20 μg/mL of proteinase K for 20 min. Embryos were rinsed repeatedly in PBT to stop the digestion and were moved into prehybridization (see below). Embryos were stored until use at the methanol step or in prehybridization at –20°C for fewer than 10 d. Embryos stored for more than 10 d showed considerable decrease in hybridization signal, especially with miRNA.

In Situ Hybridization

Prepared embryos were transferred to a standard prehybridization solution (50% formamide, 5× sodium chloride sodium citric acid (saline sodium citrate, **SSC**), 2% blocking powder, 0.1% Tween-20 or Triton X-100, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (**CHAPS**), 50 μg/mL of yeast RNA, 5 m*M* EDTA, 50 μg/mL of heparin, diethylpyrocarbonate water). Prehybridizations were for 2 h in 24-well plates (1 mL/well) in a shaking hybridization oven at 65°C for mRNA and at a temperature between 21 and 23°C below the reported melting temperature of the LNA for miRNA. Recent work indicates that up to a 5°C spread in annealing temperature (20 to 25°C below the melting temperature) is consistent with hybridization of LNA to miRNA (Kloosterman et al., 2006). Probe was added to 1 mL of fresh

prehybridization buffer, and hybridization occurred overnight at the prehybridization temperature. Embryos were transferred after hybridization to 6- or 12-well plates containing 15-mm or 24-mm Netwell Inserts, respectively, with attached 74 u*M* polyester mesh bottoms (Cat. No. 3477, 3479, Corning Inc., Corning, NY) in 2× SSC, 0.1% CHAPS prewarmed to the hybridization temperature. Inserts helped maximize wash volume and minimize embryo handling and damage for high-throughput screening. Prewarming the wash solutions to the hybridization temperature before washing was crucial for maximum signal to background ratio and is not available using some robots. Embryos in the Netwell inserts could be moved quickly into plates filled with prewarmed wash buffer, minimizing cooling for high throughput processing. Embryos were washed 3× 20 min in the high salt wash, then 3× 20 min in 0.2× SSC, 0.1% CHAPS. Embryos were rinsed twice in KTBT (50 m*M* Tris, pH 7.5, 150 m*M* NaCl, 10 m*M* KCl, 1% Triton or Tween, distilled water) and transferred back into clean 24 well plates to minimize volume for the antibody step. Embryos were pretreated in 20% sheep serum in KTBT at 4°C for 2 to 3 h or longer. Anti-DIG antibody binding (1:2,000-1:4,000) was carried out in 24-well plates at 4°C on a nutator. Final washes were in KTBT in large Netwell inserts at room temperature for a minimum of 5 changes over 5 h, but often including overnight at 4°C. Embryos were shifted back to 24 well plates into NTMT (2 solutions changes × 10 min). Color reactions (5-bromo-4-chloro-3′-indolyphosphate p-toluid ine/nitro-blue tetrazolium chloride) were for 1 to 6 h at room temperature on a nutator until signal or background became visible, followed by overnight washing in KTBT. A second or third round of color reaction followed until each probe had yielded strong signal or until the negative control began to show background label. Reactions were stopped with KTBT, and embryos were then washed in PBS, dehydrated by a methanol series to remove background and enhance signal, then stored in PBS plus 0.1% sodium azide.

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