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FORMATION OF THE NEURAL TUBE EPITHELIUM BASEMENT MEMBRANE

DURING SECONDARY NEURULATION IN THE CHICK EMBRYO

A Thesis

Submitted

In Partial Fulfillment

of the Requirements for the Degree

Bachelor of Arts in Biology with Biomedical Honors Research Emphasis

and the Requirements for the Designation

University Honors with Distinction

Leslie Anne Mataya

University of Northern Iowa

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This study by: Leslie Anne Mataya

Entitled: Formation of the Neural Tube Epithelium Basement Membrane during Secondary Neurulation in the Chick Embryo

has been approved as meeting the thesis requirements for the:

Degree of Bachelor of Arts in Biology with Biomedical Honors Research Emphasis

Designation of University Honors with Distinction

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Date Jessica Moon, Director, University Honors Program

ABSTRACT

Neurulation, the formation of the neural tube, is an important process in the development of vertebrates. Because this organ ultimately becomes the central nervous system of the organism, the proper development of a fully-functioning neural tube is paramount to its survival. Neural tube defects, like spina bifida and anencephaly, are among the most common congenital malformations present in live human births. The prevalence of these defects is a definite concern to medicine and human health.

Neural tube formation takes places via two different mechanisms: primary and secondary neurulation. Secondary neurulation, the focus of this study, takes place in the tail bud of the chick embryo. Specifically, basement membrane formation was studied in chick embryos at Hamburger and Hamilton stages 14 and 18 (approximately 50 and 68 hours of incubation, respectively). Mesenchymal cells present in the tail bud polarize to form the neural tube epithelium, and a basement membrane is deposited sometime during this process. To study basement membrane formation, the large glycoproteins fibronectin and laminin (which are present in and near basement membranes) were localized using antibodies B3/D6 (anti-fibronectin) and 31/31-2 (anti-laminin).

Results showed that laminin does not change its pattern of deposition through secondary neurulation, and is most concentrated at the perimeter of the mesenchyme and neural tube. This suggests that laminin may be involved in setting the boundary of the mesenchyme and neural tube. Its directional deposition may also allow the mesenchymal cells to polarize and adhere to the basement membrane formed by laminin to form the secondary neural tube. Although fibronectin was found surrounding the secondary neural tube, it was more concentrated in the neural crest cell region. Also, a large amount of fibronectin was involved in random cavitations in the mesenchyme, not associated with the neural tube, suggesting that it may attract cells and allow them to connect via cell junctions. Another finding was that segregation and coalescence of cavities may not always occur during secondary neurulation, as was suggested by previous research on the topic.

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INTRODUCTION

Neurulation, the formation of the dorsal hollow nerve cord or neural tube, is a complex and important part of vertebrate development. Once fully developed, the neural tube ultimately becomes the brain and spinal cord of the organism: its central nervous system. Due to its extremely important role, the appropriate development of a functioning central nervous system is vital to the survival of the organism. Consequently, if neurulation does not proceed normally, devastating effects can result.

Neural tube defects, such as spina bifida and anencephaly, are among the most common congenital malformations in humans, affecting 0.5–2.0 per 1000 live births (Saitsu & Shiota, 2008). Many more neural tube defects occur in embryos that never reach birth and spontaneously abort, ultimately resulting in miscarriages. The prevalence of these malformations is a definite concern to both modern medicine and human health. Investigating how the brain and spinal cord develop can shed light on why and how these deformities occur, and can hopefully lead to the development of preventive measures and/or treatments in the future.

Because of moral and ethical restrictions, research on human embryos *in situ*, or in the womb, is not a possibility. In view of these limitations, biologists must find alternative ways to study human processes in other vertebrates, such as chicks or mice. Although not all vertebrates form their neural tube in exactly the same way, the developmental mechanisms involved in these processes have been discovered to be very similar, the result of common evolutionary mechanisms in vertebrates.

Evolutionary developmental biology is a fascinating field that puts forth evidence for the apparent homology, or similarity, in the cellular and molecular mechanisms involved in organogenesis and body plan development. Figure 1 is a photograph by Michael Richardson, depicting the development of five different vertebrates. The top row shows earlier stages of development, while the middle and bottom rows show chronologically later stages. The
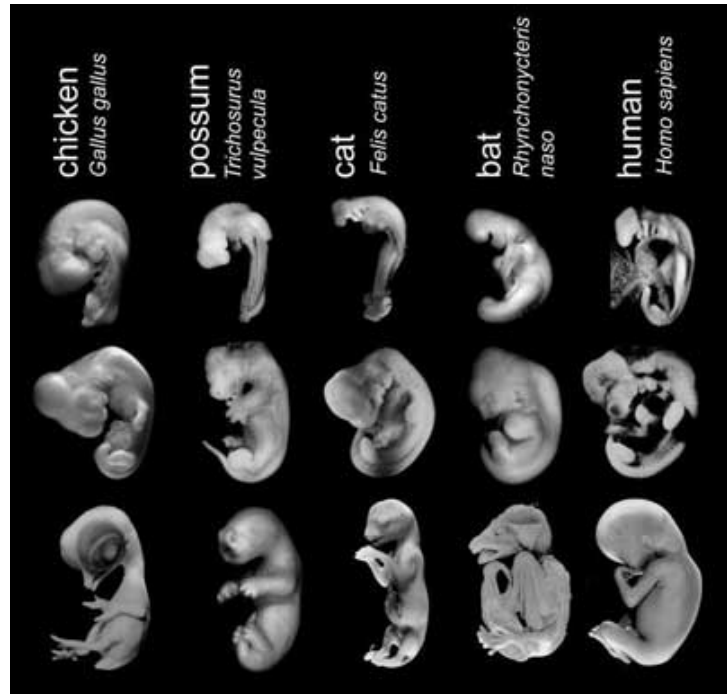
Figure 1. The Phylotypic Stage of Development (Richardson, 1997). Photograph depicting the development of vertebrate embryos. From left to right: chicken, possum, cat, bat, and human.

top row of embryos is what is known as the "phylotypic stage" of development: the stage at which vertebrate embryos most resemble one another. There are differences in the embryos at this stage, however there is a unity in organization that cannot be ignored. The phylotypic stage is the one where many major vertebrate features, including the neural tube, are formed. Due to the parallels among developmental processes in these organisms, studying neurulation in other vertebrates can give biologists a better understanding of how the human neural tube is formed.

Neural tube development in chicks, and in humans, takes place through two different processes: primary and secondary neurulation. Primary neurulation occurs in the more anterior (toward the head) regions, while secondary neurulation occurs in the more posterior (toward the tail) regions. The location where secondary neurulation takes place may also be referred to as the

"tail bud" throughout this thesis. Although both primary and secondary neurulation result in a continuous neural tube, the mechanisms by which this tube is formed are very different in these two processes. The differences between them will be discussed in more detail in the review of literature section of this thesis. Primary neurulation has been extensively studied by biologists, however, secondary neurulation is less understood. Much more investigation needs to be done on this important developmental process in order to fully understand neurulation in its entirety.

Generally, mammals with long tails, such as mice, form their secondary neural tube in a certain way, while chicks form their secondary neural tube in a different, more complex way. These processes are discussed in more detail in the review of literature section of this thesis. As stated above, human embryos also undergo secondary neurulation. Due to the fact that humans do not possess tails, they are thought to undergo the same secondary neurulation process as chicks do, rather than mice. However, biologists debate this detail. Because secondary neurulation may be similar in chicks and humans, chicks are advantageous in attempting to study the best representation of what may go on during human secondary neurulation.

During neurulation in the tail bud of the chick, cells undergo a mesenchymal-epithelial transformation through cavitation and coalescence of cavities. The tail bud starts out as a medullary cord, a large mass of loosely connected cells, without apparent organization. Small cavities appear in the mesenchymal tissue, and these small cavities combine to form the continuous lumen of the secondary neural tube. Throughout the process, these cells go from an unorganized mesenchyme to a polarized and organized epithelium. Mesenchymal-epithelial interactions and transitions, and the role of the extracellular matrix during these processes are interesting areas of developmental biology that need to be explored further.

The neural tube is epithelial tissue, which is classified as tissue that lines the cavities and surfaces of organs, glands, and blood vessels. Every epithelium, including the neural tube, has a basement membrane associated with it. The epithelial cells are polarized into basal and apical faces. In the case of the neural tube, the basal face is the outer face, while the apical face is the lumenal face. The basal face is the one that is associated with the basement membrane. This membrane is essential to the epithelium in that it acts as a mechanical barrier and a scaffold to which the epithelial cells adhere. This structure is also important during embryonic development because it aids cells in movement, organization, and differentiation into separate tissues. Due to its developmental importance, the basement membrane formation of the neural tube is the focus of this thesis. Laminin and fibronectin, glycoprotein molecules present in and near basement membranes, respectively, are fundamental molecules of this extracellular structure, and vital to its role in development.

Purpose

The purpose of this research project was to study the formation of basement membrane of the neural tube epithelium during secondary neurulation in the chick embryo. Chick embryos were harvested at various stages in development and taken through the immunolocalization procedure. Immunolocalization is the process of using antibodies to stain for certain molecules. The molecules isolated in this study were fibronectin and laminin, important markers both associated with basement membrane formation. The antibodies used were B3/D6 and 31/31-2 for fibronectin and laminin, respectively, and diaminobenzidine was used to visualize the molecules with a red stain. Immunolocalization was done in order to study the deposition of these molecules and explore their role in basement membrane formation during secondary neurulation.

Hypothesis

This thesis will explore neurulation, specifically secondary neurulation, in the chick embryo and attempt to answer the following questions:

1. What roles do fibronectin and laminin play in the formation of the secondary neural tube?

2. By what stage in development does the secondary neural tube basement membrane begin to form, and when is its formation complete?

3. How is the deposition of laminin and fibronectin different between the mesenchymal arrangement of cells and the completed neural tube?

REVIEW OF LITERATURE

Early Development of Chick Embryos

The development of any embryo begins with fertilization: the union of sperm and egg. Fertilization of the very large, yolk-filled chicken egg occurs in the oviduct of the hen, before the albumen (egg white), shell membranes, and shell are secreted to cover it. Cleavage is the next step in embryo development. The embryo undergoes rapid and incomplete meroblastic cell division, but there is not an increase in its overall volume. A hollow disk of cells known as a blastodisk is formed, with a fluid-filled cavity known as the blastocoel in the center. Once the hen has laid her egg, the blastodisk contains about 20,000 cells, and the embryo continues to develop (summarized by Gilbert, 2006).

Gastrulation follows cleavage and is one of the most important events in development: it determines the basic developmental pattern of the chick embryo. A significant amount of cell movement and rearrangement happens during this stage, leading to the formation of three primary germ layers from which all the tissue in the embryo is derived: the ectoderm, mesoderm, and endoderm. In addition to the three germ layers, the gut tube and notochord are formed by the end of gastrulation. Even before gastrulation is complete, the dorsal hollow nerve cord, or neural tube, begins to form via the processes of primary and secondary neurulation, which are described below in greater detail (summarized by Gilbert, 2006).

Primary Neurulation

Primary neurulation, illustrated in Figure 2 (next page), is induced by the already-developed notochord (Spemann & Mangold, 1924), and forms the anterior portion of the neural tube, from the cranial region to the hind limbs. It begins with the formation of the neural plate:

the notochord secretes growth factors that signal the ectodermal cells above it to thicken and elongate (Figure 2A). This elongation of cells continues posteriorly, lengthening the neural plate down the midline of the embryo (Colas & Schoenwolf, 2001).

Figure 2. Primary Neurulation in the Chick Embryo (Gilbert, 2007*)*

As the neural plate lengthens, it narrows itself so that bending will form a tube, rather than a sphere. Medial hinge point cells (MHP's) form at the midline of the neural plate, and anchor themselves to the notochord beneath them to form a furrow (Figure 2B). After the medial furrow is formed at the midline, dorsolateral hinge point cells (DLHP's) form lateral to the midline. Like the MHP's, these cells anchor themselves to the surface ectoderm that is not part of neural tube formation, forming two more furrows on either side of the midline (Figure 2C). All three of these furrows (one medial, two lateral) will direct the cells forming the neural tube and allow them to rotate into the tube formation. To continue the bending and shaping of the neural plate, the surface ectoderm (to which the DLHP's are anchored), pushes the DLHP's medially to assist with bending (Colas & Schoenwolf, 2001).

The bending of the neural plate will eventually lead to a point where the right and left neural folds will meet at the midline and join to form the neural tube (Figure 2D). When the edges of these neural folds meet, cells called neural crest cells begin to disengage from the neural epithelium. These cells are very important because they migrate to form the peripheral nervous system, head cartilage, pigment cells in the skin, and many other cell types. Because they are so

significant, some developmental biologists call these neural crest cells the "fourth germ layer" (Hall, 2000).

Secondary Neurulation
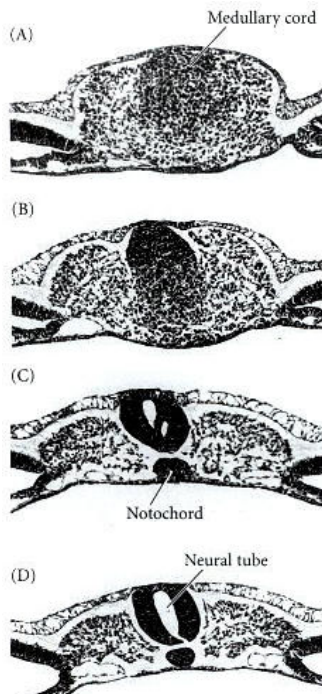
Figure 3. Secondary Neurulation in the Chick Embryo (Catala, 1995)

The portion of the neural tube posterior to the hind limbs is formed by secondary neurulation, which is seen as somewhat of a continuation of gastrulation. One way that secondary neurulation is different from primary neurulation is that it is simultaneous to notochord development, rather than induced by it (Schoenwolf and DeLongo, 1980). As in primary neurulation, cellular inductions have been found to play a role in secondary neurulation (Osorio et al., 2009).

Secondary neural tube development begins with mesenchymal differentiation and the aggregation of the caudal cell mass into a solid medullary cord (Figure 3A & 3B). The cells comprising the cord divide and form two different populations: central and peripheral. The peripheral populations undergo a mesenchymal to epithelial transformation, forming the neuroepithelium, while the central population remains mesenchymal. Cavities start to appear between the two populations of cells, which enlarge as the central cells merge with the peripheral cells by intercalation (Figure 3C and 3D). This process forms a single lumen that will become continuous with the primary neural tube. (Colas & Schoenwolf, 2001; Yang, 2003).

Some research has indicated that secondary neurulation seems to be partially dependent upon primary neurulation and the proper closure of the primary neural tube. Cavitation and coalescence may not occur properly if primary neurulation has not completed normally, and abnormal secondary neurulation can lead to malformations of the sacral spinal cord (Hall et al., 1988). Also, cavitation seems to occur somewhat randomly, and the cavities formed by this process vary greatly in size, shape, number, and location. This variation not only occurs in different embryos at the same stage of development, but at different stages of development in the same embryo (Schoenwolf & DeLongo, 1980).

The mesenchymal cells present in the medullary cord of the chick embryo are pluripotent and can give rise to many different kinds of tissues including: somites, the tail gut, and the secondary neural tube (McGrew et al., 2008). During the process of secondary neurulation, the peripheral mesenchymal cells must polarize and organize to form the pseudostratified epithelium of the secondary neural tube, which is identical to that of the primary neural tube and neural plate of earlier stages (Schoenwolf & DeLongo, 1980).

A basement membrane and other extracellular materials are laid down to begin the polarization of these peripheral cells of the medullary cord. The basal ends of the peripheral cells become interconnected by intercellular junctions and filopodia-like processes to form an *external limiting membrane*. The apical ends of these cells also interconnect via the same process to form an *internal limiting membrane* to complete the polarization process, forming a continuous neural tube (Schoenwolf & DeLongo, 1980).

Basement Membranes

A basement membrane is a "thin sheet of specialized extracellular material, typically about 50 nm thick, that underlies epithelial cells, thereby separating them from connective tissues" (summarized by Becker, 2006 pg. 489). It is the fusion of two lamina: the basal lamina and the reticular lamina, collectively known as the basement membrane. Laminin, one of the two molecules that were isolated with immunolocalization in this study, is one major component of basement membranes, including the one of the neuroepithelium. Other proteins common to all basement membranes include collagen IV, heparin sulfate proteoglycans, and entactin/nidogen. In addition to epithelial tissues, basement membranes are found surrounding nerves, fat cells, and muscle (smooth, striated, and cardiac) (Martin & Timpl, 1987).

Basement membranes have a variety of functions. They prevent passage of proteins into capillaries and glomeruli, and they maintain normal tissue formation by providing scaffolding during cell regeneration and growth. They also act as mechanical barriers and prevent malignant cells from penetrating deeper tissues.   Initially, these were the only known roles of basement membranes: as a selective barrier and scaffold for cell adherence. Over time, it has been discovered basement membrane components also play roles in cell growth, differentiation, migration, and tissue development and repair. Basement membranes are especially important during embryonic development because they allow cells to segregate and differentiate into specific tissues. In fact, it is the first extracellular matrix to appear during embryogenesis. Non-basement membrane associated extracellular matrix (containing fibronectin, the other molecule isolated in this study) is also instrumental in movement, organization, and differentiation of embryonic cells (Martin & Timpl, 1987; Erickson & Couchman, 2000).

Fibronectin

Fibronectin is a large extracellular matrix glycoprotein that mediates adhesion of cells to other extracellular matrix components. It is composed of two nearly identical polypeptide subunits linked by two disulfide bonds near the carboxyl ends of the subunits. As seen in Figure 4, each subunit has several domains that recognize and bind to one or more macromolecules present on cell surfaces or in the extracellular matrix. The RGD (arginine-glycine-aspartate) sequence is recognized by various cell surface integrins and is a common motif among extracellular adhesive proteins. Because it binds to cell surface molecules as well as extracellular matrix components, fibronectin functions as a bridging molecule that connects the cells to the extracellular matrix (Proctor, 1987; summarized by Becker, 2006).
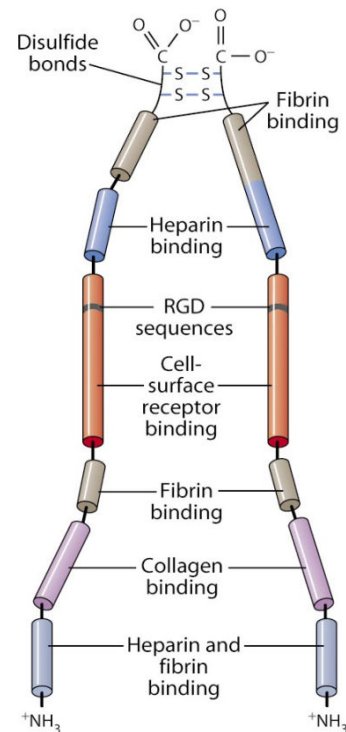
Figure 4. Fibronectin Structure (Becker, 2006)

Fibronectin can be found in a soluble form in blood and body fluids, or in an insoluble form in connective tissues and near basement membranes. Many cell culture studies have shown that large amounts of fibronectin are synthesized in several cell types, including fibroblasts, vascular endothelial cells, corneal endothelial cells, and epithelial cells of the intestine, kidney, and liver. It is thought to have a role in the organizing of the extracellular matrix and may also act as an adhesive protein for the orderly growth, migration, and positioning of cells. Other roles include maintenance of cell shape, wound healing, embryogenesis, nerve regeneration, phagocytosis, and adhesion of pathogens (viruses, bacteria, fungi) to animal cells and the

extracellular matrix. In this study, the antibody B3/D6 was used to isolate fibronectin (Mosher & Furcht, 1981; summarized by Becker, 2006).

Laminin
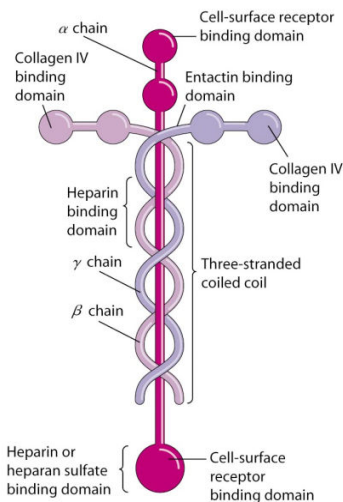
Figure 5. Laminin Structure (Becker, 2006)

Laminin, the most abundant glycoprotein present in basement membranes, is an important structural and regulatory molecule of the extracellular matrix. The molecule is a fundamental part of the structure and scaffolding in nearly every tissue in an organism, and is essential to the maintenance and survival of these tissues. It is composed of three polypeptides: denoted α, β, and γ. As seen in Figure 5, the polypeptide chains form the shape of a cross, 3 short arms and 1 long arm, all held together by disulfide bonds. In general, the 3 short arms are involved in non-cellular extracellular matrix binding, while the long arm is involved in receptor-mediated interactions, such as those with integrins (Miner & Yurchenco, 2004).

As with fibronectin, laminin contains several binding sites for collagen, heparin, heparan sulfate, entactin, and cell-surface laminin receptors. Laminin functions, as does fibronectin, as a bridging molecule, attaching cells to the basement membrane. The cross-arms on the right and left of the molecule also contain laminin binding sites. This allows the molecule to polymerize and form large sheets in order to span the basement membrane and bind to cell surface molecules. In this study, the antibody 31/31-2 was used to isolate laminin (Martin & Timpl, 1987; summarized by Becker, 2006).

The receptors that make it possible for fibronectin and laminin to bind to the surfaces of animal cells belong to a large family of transmembrane proteins called integrins. These proteins are important because they are the primary way in which the cells can bind to extracellular matrix proteins like fibronectin, laminin, and collagen. Their name describes their function: they *integrate* the cytoskeleton with the extracellular matrix (summarized by Becker, 2006).

Findings in the Mouse

Because the human, mouse, and chick are all vertebrates, neural tube formation in these species is similar, and studying this process in the mouse gives clues to how fibronectin and laminin are involved in secondary neurulation in the chick. In most mammalian species, secondary neurulation is a much simpler process than in chicks. In rodents, the secondary neural tube is formed by an extension of the primary neural tube into the medullary cord. In chicks, as reviewed above, the secondary neural tube is formed via cavitation and coalescence of these cavities into a single lumen. It is still not known whether human tail bud development follows the rodent or the chick model, and both possibilities continue to be investigated (Yang, 2003).

In 1987, K.S. O'Shea studied the deposition of these and other basement membrane components (heparan sulfate proteoglyglan and type IV collagen) during secondary neurulation in the mouse embryo. O'Shea's study used immunolocalization to isolate fibronectin and laminin, as in the present study. One difference is that O'Shea (1987) used rabbit antibodies to isolate the molecules in the mouse, while we have used mouse monoclonal antibodies to isolate the molecules in the chick embryo.

In a 10½-day-old embryo, O'Shea (1987) found scattered fibronectin staining in the unaggregated mesenchyme at the distal end of the mouse tail bud, but did not find significant

laminin staining in this region. In fact, O'Shea found that the pattern of laminin deposition was quite different from that of fibronectin during the entire process of secondary neurulation. In an 11-day-old embryo, the study found that laminin was deposited along the neuroepithelium basement membrane above the notochord, along its lateral border, but was absent in the dorsolateral region of the neuroepithelium. Fibronectin, on the other hand, completely surrounded the neural tube and was enriched in the dorsolateral region (O'Shea, 1987).

Significance of Research

Although the present study focuses on neurulation in chick embryos, this process is common in all vertebrates. All vertebrates appear to show a similar developmental mechanism for primary neurulation (summarized by Gilbert, 2006), however secondary neurulation has not been studied as thoroughly (Schoenwolf & DeLongo, 1980). As mentioned above, it is still uncertain whether human posterior neural tube development is similar to secondary neurulation in chicks, but it is very likely that the same molecules are involved in both processes.

Neurulation is a complex and important part of vertebrate development and problems during this process can lead to severe neural tube defects. These defects initiate at points in primary or secondary neurulation where the neural tube fails to form properly. According to Saitsu and Shiota (2008), neural tube defects are among the most common human congenital malformations, affecting 0.5–2.0 per 1000 live births. Many more occur in embryos that never reach birth, and spontaneously abort. Study of neural tube formation is very important to medicine, given the prevalence of these spinal cord malformations.

When anterior portions of the neural tube fail to close, a fatal condition known as anencephaly can result. This condition will prevent the development of the forebrain and skull, leading to death. Failure to close posterior portions of the neural tube results in spina bifida, a
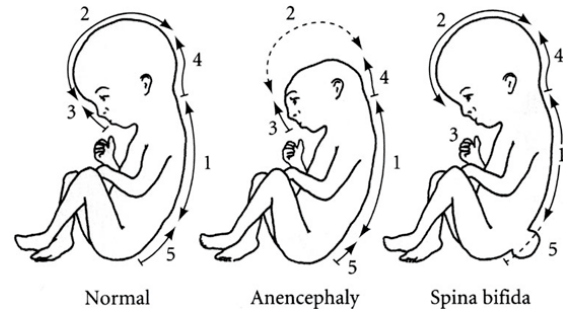
Figure 6. Neural Tube Defects (American Association of Neurological Surgeons 2004)

condition in which the spinal cord can protrude outside the body. The severity of this condition depends on how much spinal cord is exposed, and can usually be corrected by surgery. Figure 6 depicts these spinal cord malformations compared to the condition of a normal fetus (summarized by Gilbert, 2006).

MATERIALS AND METHODS

Collection of Embryos

In 1951, Viktor Hamburger and Harold Hamilton set a series of 46 chronological stages for chick embryo development: from egg-laying to hatching. Each stage has key anatomical features unique to that period in development. Figure 7 shows the Hamburger and Hamilton standard
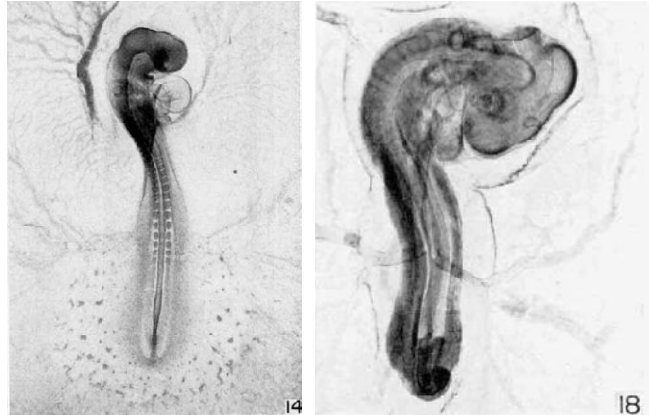
Figure 7. Photos of embryos at Hamburger and Hamilton stages 14 & 18. (Hamburger & Hamilton, 1951)

appearance of the embryos at stages 14 and 18, the stages used in this study. Fertilized chicken eggs were purchased from Sunray Chicks Hatchery in Hazleton, Iowa, and incubated at 38°C to the proper Hamburger and Hamilton stage. Incubation times per stage were determined empirically, and were approximately as follows: 50 hours for stage 14 and 68 hours for stage 18.

The egg and dissecting tools were rinsed in 70% ethanol before harvesting to ensure sterility and deter bacteria. The egg's shell was punctured with forceps, and the top of the shell was removed to expose the embryo, yolk sac and albumin. Kimwipes were used to absorb excess thin albumin, and a Whatman 3 mm filter paper ring was placed over the embryo. Scissors were used to free the embryo from the yolk. The embryo (secured in the paper ring) was then removed from the egg and placed in a 60mm dish with Chick Ringer's saline. The embryos were placed under a dissecting microscope to remove membrane and yolk and to ascertain the proper Hamburger and Hamilton stage by identifying key anatomical features on the embryo and comparing them to standard photographs (see Figure 7).

The embryo was then removed from the paper ring and placed in another 60mm dish with 4% paraformaldehyde fixative on ice. This was done to stabilize the embryos' tissues. After remaining in the fixative for approximately 20 minutes, the embryos were then transferred to a 60mm dish of 70% ethanol for storage. They were stored in this solution indefinitely.

Dehydration and Embedding

The embryos were dehydrated in an ethanol series and then embedded in paraffin wax so they could be sectioned in order to afford detailed tissue study of the tail bud and secondary neurulation. To start the dehydration procedure, the embryos (still in a 60mm dish) were submerged in a series of ethanol washes with increasing concentration (50%, 70%, 95%, and two washes of 100% ethanol) for 5 minutes each. After these steps, they were transferred to a glass container for the rest of the dehydration/embedding procedure.

The embryos were then submerged in a 1:1 ethanol:Protocol solution for 5 minutes, followed by a 15-minute incubation in the embedding oven at 55-58°C in 100% Protocol solution. The embryos were then incubated overnight in a 1:1 Protocol:paraffin wax solution, followed by an overnight incubation in 100% paraffin wax, both in the embedding oven. In order for smooth transfer of the paraffin wax, care was taken make sure the Pasteur pipettes used for transferring the wax in and out of the glass containers were incubated as well. This ensured that the pipettes were at the same temperature as the wax, and the wax would not harden inside the pipette.

Sectioning

Once completely embedded in the wax, the embryos were taken out of the embedding oven to allow the wax to harden. Using a razor blade, a metal spatula, and a Bunsen burner, the

paraffin-embedded embryo was trimmed and mounted (with the tail up) onto a wooden block for sectioning. A trapezoid-shaped block face was created to ensure smooth and straight sectioning. Sectioning was done with a Reichert-Jung 820 microtome set at 6-7 microns for each section. Before sectioning was started, the razor blade was inspected under the microscope for nicks to ensure that the ribbons would not be damaged when sectioning commenced.

The slides on which the sections were placed were covered with an adhesive known as Histogrip (Invitrogen Inc., San Francisco, CA).   Histogrip allowed the sections to remain on the slide after the wax has been removed. The Histogrip-covered slides were placed on a slide warmer at 42-43°C, and the temperature of the warmer was carefully monitored so that it did not exceed 45°C. Distilled water was boiled to remove air bubbles and allowed to cool. The water was then applied to the slides with a Pasteur pipette. The ribbons of sections were placed on the slide and arranged in an orderly fashion. Once the slide was covered with sections, the water was drawn off with a Pasteur pipette and the slide was allowed to dry while still on the warmer. This assured proper adhesion of the sections to the slides.

Rehydration

After the sections were dried on the slide, the paraffin wax was removed and the sections rehydrated, in order to leave the sections intact on the slide. The slides were sequentially submerged in a series of Coplin jars containing of Protocol, 1:1 ethanol:Protocol, decreasing concentrations of ethanol, and distilled water. Finally, the slides were incubated at 37°C in a 1N HCl solution to promote antigen retrieval. They were then rinsed in phosphate-buffered saline (PBS).

Immunostaining

In order to identify the fibronectin and laminin present in tail bud tissue during secondary neurulation, immunolocalization with monoclonal antibodies B3/D6 (anti-fibronectins) and 31/31-2 (anti-laminins) (both procured from the Developmental Studies Hybridoma Bank, Iowa City, IA) was used. Throughout this procedure, an Invitrogen Kit containing the necessary reagents to complete immunostaining was used (procured from Invitrogen Inc., San Francisco, CA). First, the slides were submerged in peroxidase quenching solution before the immunostaining procedure. This was done to prevent ambiguous background staining due to endogenous peroxidase activity. Once that step was complete, the slides were submerged in diluted primary antibody (either B3/D6 or 31/31-2). Dilution of primary antibody with blocking solution (from Invitrogen Kit) was determined experimentally, but a dilution of 1:10 (primary antibody:blocking solution) yielded the best results.

Primary antibody binding was followed by submersion in biotinylated secondary antibody, then Streptavidin peroxidase conjugate (both from Invitrogen Kit), with phosphate buffered saline (PBS) washes between steps. Following the enzyme conjugate, diaminobenzidine (a color-generating agent) and hydrogen peroxide were added to the slides until a red color developed. After staining, the slides were immersed in a hematoxylin solution (from Invitrogen Kit) to provide a blue counterstain to the red staining of the primary antibody. The hematoxylin was removed with tap water and the slides were submerged in PBS, followed by distilled water.

Mounting and Analysis

GVA mounting solution (from Invitrogen Kit) was used to mount a coverslip on each slide. Two drops were placed on the sections and the coverslip was applied. A pencil eraser was

used to push the glass down and spread the GVA solution to prevent air bubbles in the slide.

Care was taken not to push on the glass too hard, due to the risk of crushing the sections. The

slides were stored flat for two hours to allow the mounting solution to dry. When the slides were

completed, they were examined with a Leica DMIRE microscope. Digital images were obtained

using IP LAB software.

RESULTS

Interpretation of Chick Embryo Sections

In all photographs of chick embryo sections presented in this thesis, two colors of staining will be seen. The red staining will represent the protein that was immunolocalized: fibronectin or laminin, depending on the photograph. The blue/gray background staining was given by hematoxylin in order to augment and better visualize the red staining, and in order to provide tissues context. Major structures in the figures will be labeled, including the notochord (NC), tail bud mesenchyme (TBM), neuroepithelium (NE), and other anatomical features.

Laminin Staining in Tail Bud of Stage 14 Embryo

Serial cross sections were obtained proceeding from posterior to anterior. Three of these are shown in Figure 8 (page 26) that are representative and depict the progressions seen. Figure 8A shows the tail bud of a stage 14 embryo, stained with anti-laminin (antibody 31/31-2). The tail bud mesenchyme contains light laminin staining in the mesenchymal interior, while there is concentrated laminin staining toward the perimeter of the mesenchyme. This staining is located more in the ventral region of the mesenchymal perimeter. There is much less staining in the dorsal region where neural crest cells will later emerge. The red staining pattern in this figure seems to be surrounding the cells rather than inside them. This suggests that the laminin present in this section is part of the extracellular matrix outside the epithelial cells rather than being located within the cytosol.

Figure 8B is a second section from the same stage 14 embryo, but anterior to the previous section. This photograph continues the staining pattern seen in 8A, showing much more concentrated laminin staining toward the perimeter of the mesenchyme rather than in its interior.

Rather than being concentrated around the ventral perimeter (as seen in the previous section), laminin is now present all around the mesenchymal perimeter. This perimeter staining is not continuous in this figure, suggesting that laminin is not yet present in an uninterrupted basement membrane formation around the neural tube epithelium. Also, segregation and polarization of cells are starting to occur in this section, and a neural tube lumen appears to be present in rudimentary form.

Figure 8C shows the final section from this stage 14 embryo, anterior to the previous two sections. The neural tube lumen is larger and more pronounced, as is the polarization of the cells into epithelial tissue around the lumen. The perimeter staining is more concentrated and continuous than that seen in the previous sections. This suggests that laminin is present in an uninterrupted basement membrane around the basal face of the neural tube epithelium. It is still absent, however, from the more dorsal regions where there is still no clear demarcation of a border. Cell shape suggests continuing mesenchymal character.

Because of the large amount of this tissue dorsal to the lumina in photographs 8B and 8C, we hypothesize that these sections may be a part of what is called the "overlap zone." The overlap zone is a segment of the neural tube where the two neural tubes formed via primary and secondary neurulation overlap during development (Schoenwolf & DeLongo, 1980). As stated in the literature review, primary and secondary neurulation result in *one* continuous lumen. In the overlap zone, the primary lumen is formed dorsal to the secondary lumen and they both coalesce to form the continuous lumen later in development. We hypothesize that if sectioning was continued anteriorly in this embryo, a primary neural tube lumen would appear in the dorsal mass of cells, and then coalescence of the two lumina would be seen later in the development of this embryo.

Fibronectin Staining in Stage 14 Embryo

Although not shown in a figure, a different slide of the same stage 14 embryo was stained with B3/D6 (anti-fibronectin) and was examined in order to compare the pattern of fibronectin and laminin during secondary neurulation. Polarization and formation of epithelial tissue are observed, along with the segregation of cells to create multiple cavities, which would eventually coalesce and form the secondary neural tube later in development. Unlike laminin, which was present on the perimeter of the mesenchyme in the figures, fibronectin appears among cells interior to the perimeter. This inward location is more significant than what was seen in the laminin sections. Also, the fibronectin staining is concentrated more in the dorsal region of the neural tube. This concentration of staining may accompany the initiation of neural crest cells which emerge from an epithelial to mesenchymal transformation on the dorsal side. Due to limitations of our technique, only one section was visualized with anti-fibronectin staining. Thus the results are not conclusive with only one section to provide data.

Fibronectin Staining in Stage 18 Embryo

Figure 9 (page 26) shows a section through a stage 18 embryo, in a more anterior region of the trunk neural tube, where primary neurulation takes place. We know that this section is a product of primary neurulation, because a notochord is present. As stated in the literature review, primary neurulation is induced by the notochord, whereas secondary neurulation is not. In this photograph, fibronectin is found abundantly around the notochord, the dorsal aortae, and along the dorsolateral aspect of the dermamyotome. Around the neural tube, however, the staining is not as smooth or intense. Fibronectin is present at less intense levels throughout the mesenchyme and has a more fibrillar form in this area of the section. Interestingly, fibronectin staining is concentrated at the most dorsal aspect of the neural tube, where neural crest cells are

beginning to go through epithelial/mesenchymal transitions. All of these locations for fibronectin are well known during primary neurulation, and our observations are in accord.

Sequence of Secondary Neurulation in Stage 18 Embryo (Stained with Fibronectin)

All of Figure 10 (page 27) shows a sequence of five sections taken from a series of twenty from the same stage 18 embryo. The sequence starts from the tail bud and moves anteriorly. Figure 10A shows mesenchymal tissue at the posterior tip of the tail bud, not yet differentiated into neural epithelium. Note that this image is magnified 20X, rather than 10X. In the photo, interior, localized areas of fibronectin staining can be seen. The interior areas of staining appear to be somewhat random in pattern, and there is very little staining on the perimeter of the mesenchyme. The most intense fibronectin staining in this section appears to be intracellular, rather than extracellular suggesting that fibronectin is present in the cells but not yet secreted into the extracellular matrix. These cells may be in the process of secreting fibronectin. However, the relative amount of fibronectin staining compared to the other sections in this series is very little.

Figure 10C shows a section anterior to the previous one, and features a small neural epithelium surrounded by mesenchyme. There is also a small cavitation present in the middle of the neural epithelium. We noted that only *one* cavity was seen during the development of this secondary neural tube. We did not observe multiple lumina in this embryo. The staining pattern in the mesenchyme is present in the same random pattern seen in the previous section, and is not seen in abundant amounts in this region. The most fibronectin staining seen in this section is associated with the small neural epithelium, not the mesenchyme. It is associated with the peripheral areas of the epithelium, and hardly any staining is seen in the interior among the epithelial cells. The staining is also not tightly arranged around the neural tube epithelium.

In Figure 10D, a more anterior section is shown. The neural tube epithelium is seen to be more pronounced, and the lumen of the neural tube is larger. Light, diffuse fibronectin staining is seen around the neural tube, and the arrangement of fibronectin in this region is fibrillar. The concentrated fibronectin staining present in the mesenchyme is in a jagged, linear pattern ventral and bilateral to the neural tube. Small, irregular lumina are visible in the mesenchyme, but their pattern seems random and not necessarily indicative of segregation and formation of neural tube epithelial tissue.

Figure 10E shows another section that is more anterior. Here an even larger and more vertically elongated secondary neural tube is seen. Again, cavitations are seen in the mesenchyme, but they still do not appear to be a part of developing neuroepithelium. The most concentrated fibronectin staining is in this region where the lumina are forming in the mesenchymal tissue. As seen in the previous section, the lumina and staining around them appear to be random. There is light staining around the neural tube, and it is more concentrated in the dorsal, neural crest cell region.

In Figure 10F, showing the final section in this sequence, the neural tube is the largest and most elongated seen. The cloaca is now visible directly ventral to the neural tube. Staining is again seen in the dorsal region of the neural tube, where neural crest cells are located. Between the neural tube and the mesenchyme, light, fibrillar fibronectin staining is seen, as was in previous sections. The lumina are still observed in this section with intense staining around them, but their formation still does not appear to be a part of any developing neural epithelium. Small, dense collections of mesenchymal cells are seen on either side of the neuroepithelium, with intense fibronectin staining.
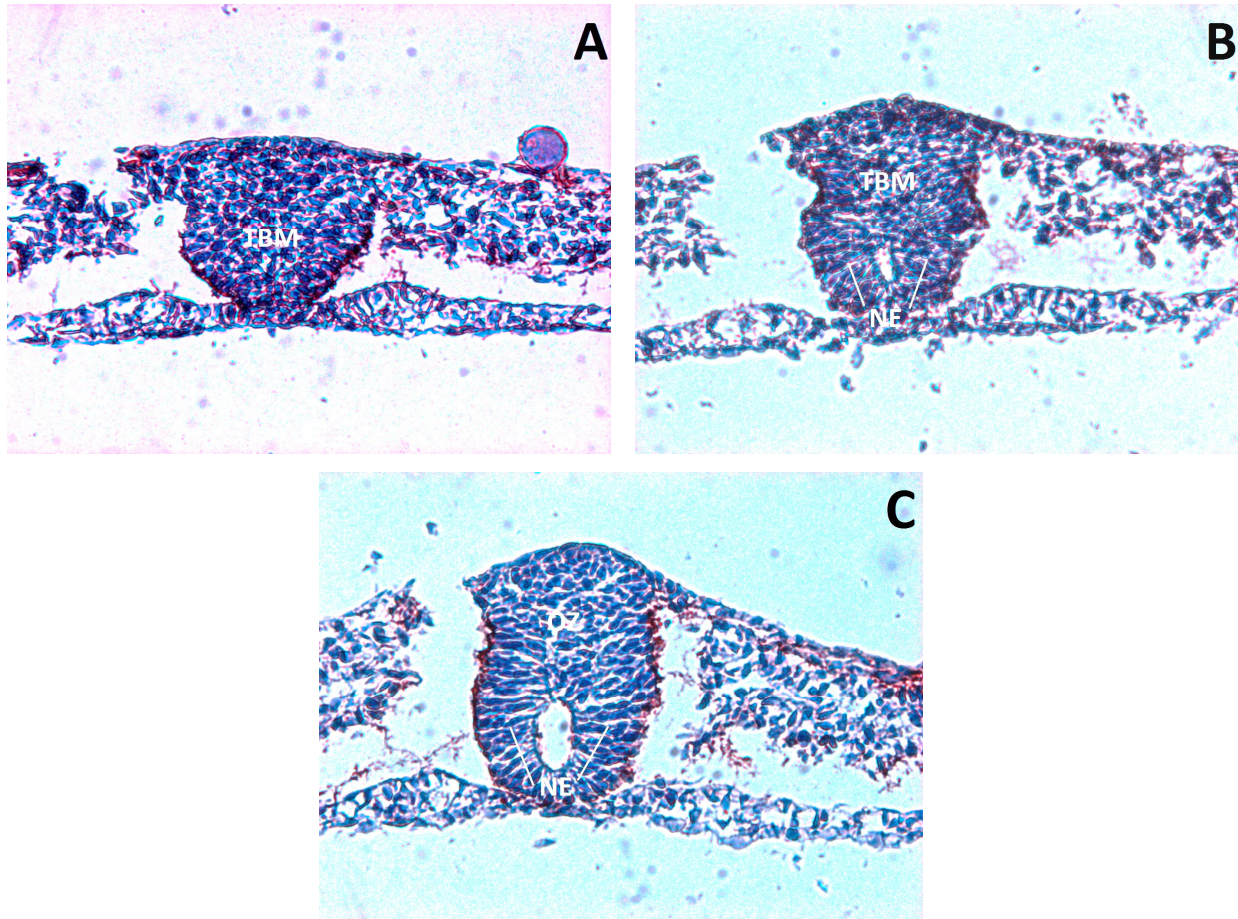
Figure 8. Laminin Staining in Secondary Neurulation (Stage 14). *(A)* Light, interior mesenchymal staining; more concentrated staining on ventral perimeter of tail bud mesenchyme (TBM). *(B)* Concentrated, not continuous, laminin staining around entire mesenchyme; small neuroepithelium forming (NE). *(C)* More pronounced neuroepithelium; continuous laminin staining around neural tube; overlap zone (OZ) dorsal to neural tube lumen.
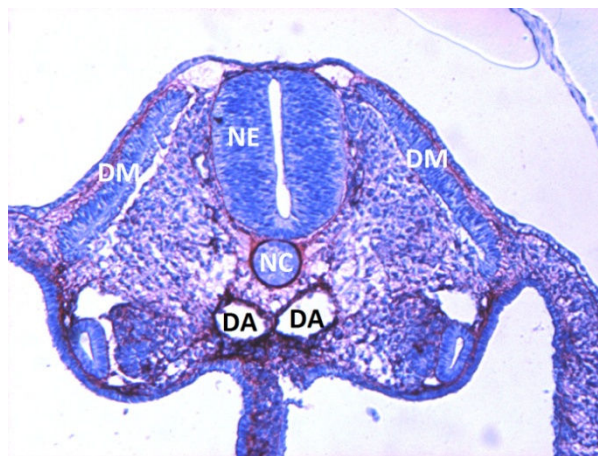
Figure 9. Fibronectin Staining in Secondary Neurulation in (Stage 18). Well-formed neuroepithelium (NE) with increased staining in dorsal neural crest cell region. Intense staining around notochord (NC), dorsal aortae (DA), and dorsolateral aspect of dermamyotome (DM).
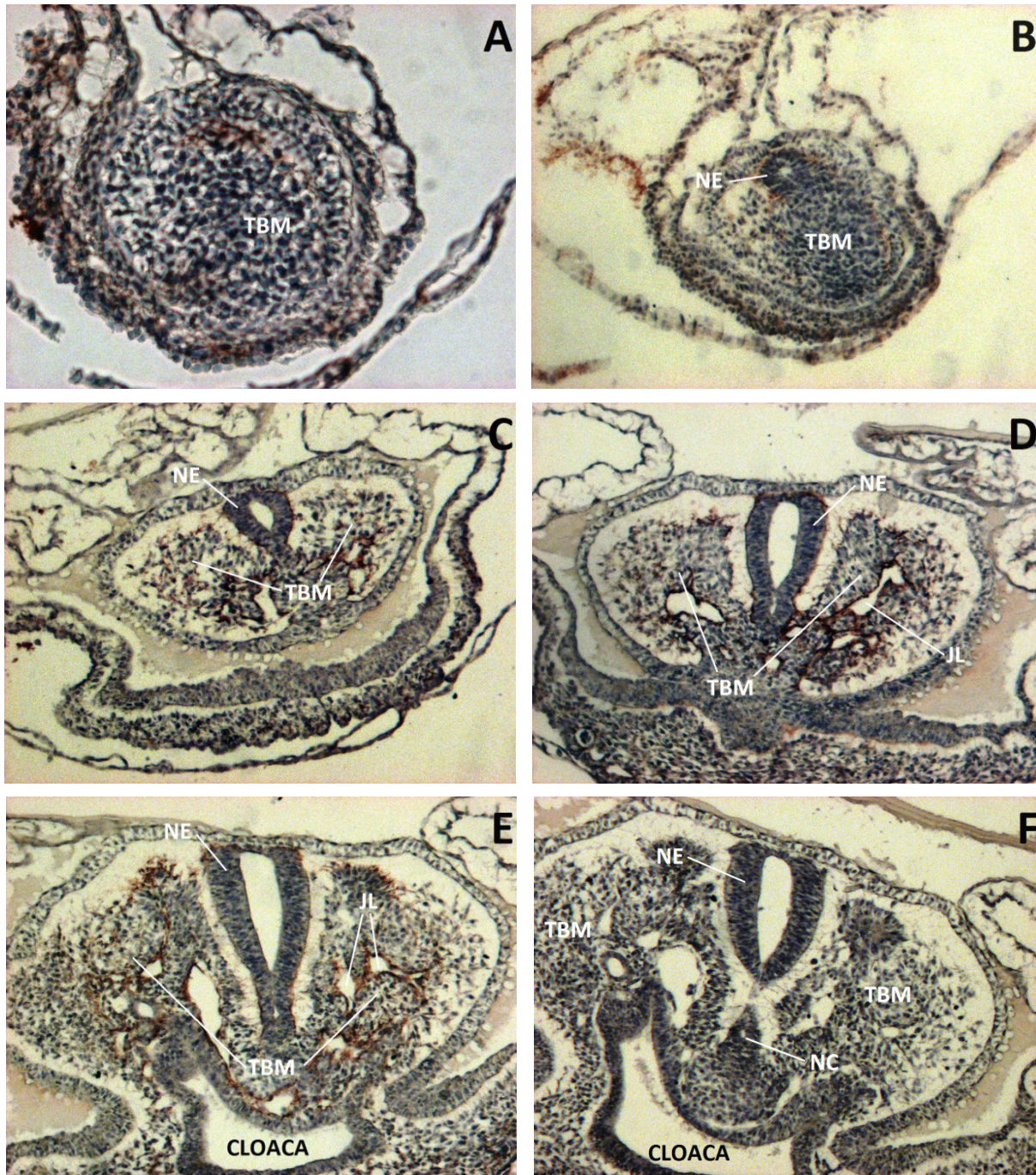
Figure 10. Secondary Neurulation Sequence from Posterior to Anterior (Stage 18). *(A)* Tail bud mesenchyme (TBM) magnified at 20X. *(B)* Small, neuroepithelium (NE) with intense fibronectin staining. *(C)* Larger neuroepithelium with intense fibronectin staining in the mesenchyme. *(D)* More developed neuroepithelium, with intense fibronectin staining surrounding jagged lumina (JL) in the mesenchyme. *(E)* Vertically elongated neuroepithelium with fibronectin staining in the dorsal neural crest cell region. *(F)* No antibody control, rounder neuroepithelium (NE), and possible notochord aggregation (NC).

DISCUSSION

Role of Laminin in Secondary Neurulation in Chicks

As discussed in the review of literature, laminin is an extracellular glycoprotein that is important in structure and scaffolding in nearly all tissues. These molecules can polymerize and form basement membranes that are found at the basal surfaces of all epithelial tissues. In addition to many other functions, this basement membrane provides a scaffold to which the epithelial cells adhere and are able to organize.

In comparing our results with O'Shea's study (1987) on mouse embryos, there are some similarities between the mouse and the chick deposition of laminin during secondary neurulation. In the pattern of laminin staining in the mouse embryo, O'Shea found that laminin was deposited along the neuroepithelium basement membrane above the notochord. The study also found very little staining in the dorsal, future neural crest cell region. These findings are consistent with ours: we found more staining in the ventral and lateral regions of the neural tube and mesenchyme, and not much in the dorsal neural crest cell region. This suggests that although laminin is involved in neural tube organization, it is not involved in neural crest cell migration or differentiation.

Osorio et al. (2009) studied various extracellular matrix components, including laminin, during mesenchymal/epithelial transitions in chick embryos at stages 18-20. The laminin staining they found was spotty and discontinuous; therefore they concluded that the basement membrane was not fully formed at this point in development. This is similar to what we found. They found laminin staining throughout the perimeter of the mesenchyme, including the neural crest cell region. Our results did not show staining in this region, but because our embryo was at

stage 14 rather than stage 18, this may account for the difference. Also, because we obtained

only a few sections with staining, the pattern observed may be an artifact of that particular slide

or embryo.

Throughout the sections with laminin staining, a general pattern was apparent: laminin

was present at lower intensities in the interior of the tail bud and at concentrated levels on the

perimeter. This pattern did not change in the sections we analyzed. Thus, we conclude that

laminin is most concentrated at the perimeter of the neural tube in order to inform other cells

where the "edge" is and which way is outward. The low levels of staining present in the

mesenchymal interior show that all cells contain laminin in their extracellular matrices but not as

much as the cells that line the outside of the neural tube. Laminin marks the perimeter of the

neural tube, and the cells there apparently secrete it directionally: to the basal face of the

epithelium.

This directional secretion may also play a role in polarization of mesenchymal cells into

the epithelium. Laminin is secreted to the basal face of the epithelial tissue, where it polymerizes

with other matrix molecules to form a basement membrane. As previously discussed in the

review of literature, basement membranes provide a scaffold to which cells adhere. In the case

of the neuroepithelium, cells secrete laminin toward the perimeter of mesenchyme. This allows

the mesenchymal cells to adhere to the newly formed basement membrane, polarize, and form

the secondary neural tube. The formation of multiple lumina would necessitate a more complex

mechanism, however it seems conceivable that the basic elements are operating.

Role of Fibronectin in Secondary Neurulation in Chicks

As discussed in the review of literature, fibronectin is an extracellular glycoprotein secreted from cells and involved in organizing of the extracellular matrix, and the growth, migration, and positioning of cells. During neurulation, fibronectin is specifically involved in neural crest cell migration and the formation of the basement membrane. During the sequence of secondary neurulation studied, fibronectin was present in relatively abundant amounts in the neural crest cell region, around the neural tube, and throughout the mesenchyme.

In comparing our results with O'Shea's study (1987) on mouse embryos, there are many similarities between the mouse and the chick deposition of fibronectin during secondary neurulation. At the posterior tip of the tail bud, scattered fibronectin staining was found, and this staining was located more lateral to the midline of the mesenchyme. In further anterior regions, a continuous boundary of fibronectin staining was found surrounding the neuroepithelium. This continuous staining was especially dense at the dorsolateral border of the neuroepithelium: the neural crest cell region. Our findings were concordant with these: we found increased staining in the neural crest cell region but we also found apparently copious amounts of fibronectin in the mesenchymal regions on either side of the neural tube.

Osorio (2009) studied the deposition of various extracellular matrix components, including fibronectin, during secondary neurulation in chick embryos at stages 18-20. The pattern of staining found in these sections was very much like the one found in this study: a continuous ring around the neural tube with concentration at the neural crest cell region. This pattern suggests that fibronectin is related to basement membrane formation but more directly involved in neural crest cell migration and differentiation.

What was not clear from either of the studies by Osorio (2009) or O'Shea (1987) is the intense amounts of fibronectin staining in the mesenchyme in the more anterior secondary neurulation sections that we observed in this study. The bright red staining surrounded jagged lumina that were not ultimately involved in the formation of neural tube cavitations. These cavities seem random, and it is not known if they always form during secondary neurulation. Because only one sequence of sections was obtained for analysis, it is not clear if these cavitations and this pattern of staining always occur during secondary neurulation in chicks.

The intense fibronectin staining also had cells aggregated around it. Based on this pattern, we can propose that fibronectin may be secreted by a few of these cells, and this secretion attracts other cells to attach to it. Once enough of these cells gather on a fragment of fibronectin molecules, the cell would be close enough to connect via cadherins such as N-cadherin and cadherin-11. Both are potentially involved in secondary neurulation (personal communication, D. Wiens).

Cavitation and Coalescence May Not Always Occur during Secondary Neurulation

Previous research on secondary neurulation (Colas & Schoenwolf, 2001; Schoenwolf & DeLongo, 1980; Yang, 2003), has suggested that the secondary neural tube forms via segregation of epithelial cells, formation of two or more cavities, and the coalescence of these cavities into one continuous lumen. The amount and location of these cavities has been found to be random: ranging from just two cavities to four or five, coalescing to form the main lumen. Although this pattern of formation was seen in a couple of sections in this study, it was not seen in others.

In this research project, a sequence of secondary neurulation in a stage 14 embryo (Figure 8) was examined. Continuous sections were taken from the tail bud mesenchyme to more anterior regions of the secondary neural tube, where it was more completely formed. We note that the images in Figure 8 are not continuous sections: not all sections gathered are displayed in this thesis, but were indeed analyzed. In examining these continuous sections, the formation of only one cavity was observed rather than multiple ones. Even as the secondary neural tube got larger and more well-formed, still only one cavity was seen.

We propose therefore, that while formation of multiple cavities with coalescence occurs during the majority of chick embryos undergoing secondary neurulation, it may not *always* occur. Because the amount and location of cavities is random, and sometimes as few as two cavities have been observed, we feel that it is plausible that the secondary neural tube may form from only one cavitation. Because we observed this in only one embryo, more research must be done to see if this formation of the neural tube is commonplace.

Conclusions

In conclusion, the results of this study showed that laminin does not change its pattern of deposition through secondary neurulation, and is most concentrated at the perimeter of the mesenchymal and neural tube. This suggests that laminin may be involved in setting the boundary of the mesenchyme and neural tube. Its directional deposition may also allow the mesenchymal cells to polarize and adhere to the basement membrane formed by laminin to form the secondary neural tube. Although fibronectin was found surrounding the secondary neural tube, it was more concentrated in the neural crest cell region. Also, a large amount of fibronectin was involved in random cavitations in the mesenchyme, not associated with the neural tube, suggesting that it may attract cells and allow them to connect via cell junctions. Another finding

was that segregation and coalescence of cavities may not always occur during secondary neurulation, as was suggested by previous research on the topic.

Limitations and Future Directions of the Study

Schoenwolf and DeLongo (1980) showed that secondary neurulation begins around stage 14. Yang et al. (2003) later determined that the finalizations of secondary neurulation (coalescence and the formation of a continuous lumen) are completed by stage 35. However, in this study, only two stages of embryos were obtained for analysis (stages 14 and 18). In order to fully examine fibronectin and laminin deposition during this process, more embryos at stages in which secondary neurulation take place (stages 14–35) must be sectioned, stained, and analyzed.

As with any research, there are obstacles in collecting data, and analyzing and interpreting that data. Due to difficulty with our methods (described in more detail in Appendix A), we did not obtain as much data as we would have liked over the course of the project. As discussed in the results/discussion sections: some of our findings may be artifacts of that particular section or embryo and may not be applicable to the general process of secondary neurulation. The only way to ascertain whether our data is accurate is to carry out further experiments on additional embryos. Only then can these questions truly be answered.

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APPENDIX A – Methodological Difficulties

Throughout our research process, as in any type of scientific research, my advisor, Darrell Wiens, and I encountered many difficulties. The minor problems, like opening eggs without usable embryos in them, or not getting the right H&H stages in harvesting, or having solutions/antibodies go bad, set us back a bit but were otherwise manageable. There were other puzzlements throughout the process, however, that were not as easy to navigate around.

During the final months of this study, our protocol was first harvesting the embryos, then sectioning and mounting onto slides, and ending with immunolocalization and staining of the sections present on the slides (see Materials and Methods section). When we first started the research in January of 2010, though, our protocol was different. We started the procedure with harvesting, then immunolocalization and staining of the *whole* embryo, then sectioning and mounting onto slides after immunolocalization was complete. This treatment of the embryos, however, resulted in a bizarre shrinking effect. During the dehydration step, when we would put the embryos in 100% Protocol in the incubating oven at 55-58°C, the embryos would shrink to almost a third of their original size. This shriveling would result in undistinguishable sections, and in most cases, the tissue would fall completely out of the paraffin wax when sectioning, rendering no data.

This continually occurring result was very perplexing, one that Dr. Wiens had never seen during all his years of research with chick embryos. We tried to determine the cause of the shrinking by doing mini-experiments with spare embryos to see which part of the protocol was causing the effect, but to no avail. We changed the solutions we were using with fresh ones, but this did not work either. We also checked the temperature of the incubating oven, and found it to be exactly the temperature indicated by the immunostaining guidelines given in the Invitrogen

Kit. Something in the immunostaining procedure was causing the embryos to shrink, but we could not determine which exact step it was. Due to this dead-end at which we found ourselves, we decided to change our protocol to the one discussed in the Materials and Methods section. We secured the sections on the slides first, and *then* carried out the immunolocalization and staining procedure.

This new treatment of the embryos did yield some results, however it had its own shortcomings. The sections were able to be visualized on the slides without the previous shrinking effect, but staining was not always effective. On a single slide, there would be sections with a good amount of staining, and others with none at all. Dr. Wiens and I still cannot determine the cause of this strange staining pattern. Due to this hit-and-miss sort of approach, it was difficult to obtain sequential data to be analyzed. But, in closing, I'll quote Victor Hamburger: "Our real teacher has been and still is the embryo, who is, incidentally, the only teacher who is always right" (Holtfreter, 1968).

APPENDIX B – Reagent Preparation

Chick Ringer's Saline (1L)

9.0g NaCl

0.42g KCl

0.214g CaCl$_2$

Bring to total volume of 1L with distilled H$_2$O

4% Paraformaldehyde Fixative (100mL)

4.0g Paraformaldehyde

Add distilled H$_2$O until volume is 66.0mL (in beaker for mixing)

Heat in hot water bath or on hot plate

Titrate with NaOH until solution is clear and allow the solution to cool

Add 25mL of 4x PBS

Bring to total volume of 100mL with distilled H$_2$O

1x PBS (200 mL)

1.6g NaCl

0.04g KCl

0.288g Na$_2$HPO$_4$

0.048g KH$_2$PO$_4$

Adjust pH to 7.4 and bring to total volume of 200mL with distilled H$_2$O.

4x PBS (500mL)

16g NaCl

0.4g KCl

2.88g Na$_2$HPO$_4$

0.48g KH$_2$PO$_4$

Adjust pH to 7.4 and bring to total volume of 500mL with distilled H$_2$O.

Other Reagents – obtained from Invitrogen Kit