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Studies on the Relationship between the Embryonic Heart Development and the Amnion Folding in Chick

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Abstract: As a model animal for developmental biology, chick embryo is easy to control and observe during embryo development period and therefore it is widely used in the study of cardiac development. The application of proteomics has opened the door for large-scale studies to dissect both protein expression, regulation and function during chick heart developing stages. The proteomics study requires to quickly separate a large number of chick heart samples with the same developing stage. However, the traditional morphological standards based on Hamburger-Hamilton and Witschi stages are difficult to fulfill this requirement. Herein, we suppose a new standard for distinguishing chick heart morphology in different developing stages based on the relationship between the embryonic heart development and the amnion folding in chick. Based on this standard, we can accelerate the speed of embryonic heart sample separation and guarantee the quantity and quality of the sample more reliably.

Key words: Chick embryo, heart development, amnion folding, proteomics

Introduction

The heart is the first organ to perform its function during vertebrate embryo development (Wu *et al*., 2003). Understanding of molecular mechanisms for heart development has been a topic of inquiry. While untill now a large number of genes and their functions remain to be identified, although much progress in clarifying the genetic pathway of heart development has been made.

The chick is one of the ideal model in vertebrate research. It is a representative of polyyelk-egg animals, such as birds and reptiles and also is a transitioual type between the simple development course, such as fish and amphibian animals and the complicated development course, such as mammalian. The study on chick development will not only establish a good basis in understanding the mammal embryology, but also offer information about the mammal early development, which is hard to be expounded, especially in the case of heart development (Lamers *et al*., 1991; Levin *et al*., 1995).

Proteomics is a systematic research approach aiming to provide the global characterization of protein expression and function under given conditions. It has been widely used in developmental study (Stults *et al*., 2005). The identification of protein molecules during different developing stages with proteomic technology will help to understand the development mechanisms and thus facilitate to discovery new strategies for the therapeutic and clinical management (Gallego-Delgado *et al*., 2005).

In order to utilize proteomics to study the proteins involved in chick heart at different development stages, it is required to distinguish the subtle developmental stages of embryo

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heart rapidly and accurately. However, the two traditional morphological standards from Hamburger-Hamilton (HH) and Witschi stages (Hamburger *et al*., 1951; Harh *et al*., 1975), which based on the number and size of somite, are not suitable for the proteomic studies, because it is difficult in practical operation to rapidly and accurately separating a large number of heart tissues from embryos at different stages to meet the requirement. In order to settle the problem, we studied the relationship between the embryonic heart development and the amnion folding in chick and appraised the subtle events of every cardiac developmental stage based on the amnion folding during the chick embryo development. As a result, a new morphology standard is established to distinguish heart developmental stages rapidly and accurately, which may provide a basis for the subsequential analysis on proteomics level during cardiac development.

Materials and Methods

Materials

White fertilized chick eggs of Hainan were bought from the animal husbandry place of Hunan Province.

Experiment Methods

Incubation of the Chick Embryos

Put the fresh fertilized egg in 37°C damp-regulating temperature testing incubator. The freshness of eggs will influence the experimental result directly, so don't keep eggs at room temperature exceeding 48 h, otherwise it will effect the normal development of the embryo seriously.

Micro-observation and Separation of the Embryos

According to the scheduled hatch time, take out the eggs after proper time of incubation and prepare a clean culture dish. Open the eggs carefully and transfer the yolk and albumin into the culture dish. Pay attention not to break the yolk membrane, otherwise the flowing-out yolk will probably conceal chick embryo, which is affixed to yolk membrane. Then, cut down the yolk membrane with dissecting scissors, ladle out chick embryo carefully and clean it in 1×PBS for two times, transfer it to the culture dish which is filled with the distilled water and observe it under the stereoscope.

Separation of Embryo Heart

Firstly, using dissecting needles get rid of membrane tissues around the embryo, we can judge the developmental stage and the position and form of the heart in the center of the embryo clearly. Then cut apart the portion with the heart, move it to the ultra-pure water and strip other tissues adhering with heart. It is important to get rid of additional tissues and to keep the heart tissue untouched, which ensure the dependability of the cardiac proteins extracted in the following treatment.

Photography of the Embryos and the Hearts

Take a photograph of each chick embryo and the heart with a Nikon 3 million digital camera.

Results and Discussion

A New Method to Confirm the Chick Embryo Developmental Stage

In order to use the proteomics method to confirm embryo development incident at each developmental stage, we should first understand and describe the true events during chick heart development accurately. The traditional standards, HH and Witschi stages, are not easy to ascertain the developmental stage quickly and accurately, especially from HH14 to HH19. During

Table 1: The morphology standard of the developmental stages of chick embryo

Incubation hours	HH stage	Witschi stage	Characteristics determined by HH	Morphologic point of embryonic development observed by this study	Morphologic point of embryonic heart development observed by this study	No. of embryos observed
18-19 h	4	12	The primitive streakat later stage is ± 1.88 mm			20
19-22 h	5	13a	The head process (notochordal process)			20
23-25 h	6	13b	The head fold	The head fold begins to appear as an intact semilune pleat.		20
23-26 h	7	14a	One somite and the neural fold	1-3 pairs of somites form; the head pleat grows rapidly, obviously projecting on the blastoderm and forward over the amnion area; the neural fold forms.	The mesoderm thickens and the primitive cardiac region forms	40
23-26 h	7 -8-	14b	1-3 individual somites, body cavity	The neural fold developments obviously and the two sides are drawn close to each other.	The endocardial primordium gets closed and combined	40
26-29 h	8	14c	4 somites and the blood island	4 individual somites; The neural ditch takes shape, still keeping open; the dorsal view shows that the edge of neural folds on two sides draw close to each other, but don't meet yet and spread forward and backward; the ventral view shows the obvious semilune of the anterior intestinal portal edge	The straight cardiac tube appears	40
29-33 h	9	15a	7 somites and primitive ocular vesicle	7 individual somites; the dorsal view shows that the rostral end of the neural tube enlarges obviously compared with the caudal end and that the edge of the neural fold moves to midline; the ventral view shows that the anterior intestinal portal edge descends but still keeps the semilune shape.	The cardiac tube extends constantly, combines from the caudal end to rostral end and forms a joined cardiac tube as a result.	40
33 h	9+ to 10-	15b	8-9 somites and amniotic head fold	The neural tube continues to expand and the primary brain vesicle begins to take shape.	The cardiac tube begins to bend toward right like C	40
33-38 h	10	15c	10 somites and 3 primary brain vesicles	3 primary brain vesicles (forebrain, midbrain, water chestnut brain); the anterior neural foramen has not been closed yet; the straight cardiac tube.	The right groove fold becomes more flat, but the left groove fold deepens.	40
40-45 h	11	16a	13 somites and 5 neuromeres of hindbrain	Hindbrain has 5 neuromeres; the anterior neural foramen has healed; a pair of optic vesicles forms on the protuberance area on the side of forebrain; the heart begins to project toward right.	The ventricle awl groove appears, dividing the primitive ventricle curve from the awl body	100
45-49 h	12	16b	16 somites and fore-brain vesicle	The embryo begins to turn back; the heart protrudes toward right further.	The primitive atrium appears	100
48-52 h	13	16c	19 somites and the atroventricular canal	The angle with which the embryo turns back increases, exceeding 45 degree; the heart protrudes toward right further and the primary flowing canal is visible from the dorsal view; the amniotic head fold overlays lower edge of head.	The primary flowing canal curved toward right, which marks the end of cardiac circularization.	100
50-53 h	14	17b	22 somites, the crooked truck and the gill arches I and II, gill slits 1 and 2	The anterior half of the embryo turns to the side and the head region forms; the left side of head leans against the yolk upper edge; the caudal part of the embryo keeps unmoved, with the ventral side on yolk; the amniotic head fold moves backward reaching under the level of anterior intestinal porta and wraps the embryo with amniotic lateral fold.	The primitive atria moves from the bottom of cardiac tube to the top and next to the conus; the left atrium is located at the rostral and left side of the right atrium; the near end of primitive ventricle curve is located on the rostral and left side of the far end of primitive ventricle curve.	100

Table 1: Continued

Incubation hours	HH stage	Witschi stage	Characteristics determined by HH	Morphologic point of embryonic development observed by this study	Morphologic point of embryonic heart development observed by this study	No. of embryos observed
50-55 h	15	17d	24-27 somites, the gill arch III and gill slits 3	The brain bends to nearly overlapping, taking the midbrain as the axis and bends to nearly overlapping. Taking the midbrain as the axis, the brain folds to nearly overlapping; the upper edge of brain becomes vertical; the anterior forebrain is close to heart; the body curves a little and its outline shapes like an arc; the edge of amniotic fold is located at the middle poison of embryo, which is between the two levels of anterior intestinal porta and omphalomesenteric artery.		200
51-56 h	16	18	26-28 somites; Wing bud; amniotic caudal fold	The edge of amnioitc fold is located slightly above the omphalomesenteric artery.		200
52-64 h	17	19	29-32 somites and leg bud	The edge of amnioitc fold passes omphalomesenteric artery and continues to wrap downward; the body curves further and the lower edge of heart contacts with body.		200
3d	18	20	30-36 somites; the somites extend downwards and over the position of the leg bud; the allantois takes shape.	The edge of amniotic fold merges into a hole at caudal end; the back flexure shapes as 90 degree.	The position where the primitive ventricle is crooked moves from lying in the rostral end of the atrium to the caudal end of the atrium finally; this course is finished at HH18; during this stage, the position of left and right atriums and primitive ventricle curve changes greatly and gets close to their destined position; the left groove of the primitive atrium disappears at final stage.	200
3.0-3.5 d	19	21	37-40 somites; the somites extend into the tail; the maxilla extends.	The edge of amniotic fold totally heals at caudal end.		200
3.0-3.5 d	20	22	40-43 somites; the embryo totally turns back; melanin begins to deposit in *eyes*	The embryo totally turns back; the volume of allantois is very small.	The truncus arteriosus appears and extends; the position of primitive effusion channel (conus) relative to atrium changes; when the atriums arrive to their destined final position (located at the ventral side of left atrium), the proximal 2/3 part of the conus moves from the right site; till HH24 stage, the tube character of the primitive heart disappears; so far, the heart obtains the outline of an adult heart, but has not finish the interior separation.	200
3.5 d	21	23	43-44 somites; gill arch IV; gill slit 4	The allantois volume increases, shapes as a ball and its lower edge is tangent with the lower edge of the tail; the fore-limb bud and the hind-limb bud are obvious, protruding as hemisphere, the head and the tail are stillin a distance apart.		40
3.5-4.0 d	22	24	The somites extend to caudal end.	The allantois volume continues to increase, the fore- and hind-limb buds continues to grow and the fore-limb bud is club-shaped; the embryo curves further, so that the rostral and caudal ends get closer.		40
4 d	23	25	The back outline line from hindbrain to tail takes the form of curve.	The back outline from hindbrain to tail shapes like curve and the embryo curves so much that the head and tail contact to each other closely; the allantois is pinched to heart-shaped.		20

Table 1: Continued

Incubation hours	HH stage	Witschi stage	Characteristics determined by HH	Morphologic point of embryonic development observed by this study	Morphologic point of embryonic heart development observed by this study	No. of embryos observed
4.5 d	24	26	pedal disc	The allantois continues increasing, protrudes outwards from navel cord, forming one sizable bag with a handle; toe disc appears and the forelimb bud differentiates to palm; so far, the heart has already had the appearance like an adult heart, but the inside has not totally separated yet;		20

these stages, many important incidents emerge, such as looping of the cardiac tube, formation and expanding of the atrium and ventricle, initial beat of the heart, etc. (Garcia-Martinez *et al*., 1993; Manning *et al*., 1990; Martinsen, 2005).

To guarantee the consistency of embryo development stage and the repetitiveness and dependability of the results of proteomics assay, we have set up a new morphology standard to distinguish the developmental stages of chick embryo, especially the heart, which is mainly based on the cicatrisation degree of the amniotic border during the development stages from 48 to 75 h. The compartmentalization of this standard, the main incidents of heart development and the difference from that of HH are shown in Table 1.

The Relationship Between the Change of the Amniotic Fold and the Heart Development at Different Stages

It is undoubted that the amniotic fold change has close relationship with the key period of heart development (Eyal-Giladi *et al*., 1976), so it can be used as an important observation standard to divide the embryonic development stage and will be more accurate. Their relation is summarized as following.

At 48 h of embryo development, the cardiac tube begins to fold and loop and the amniotic edge appears in the top region adjacent to cardiac region (Fig. 1A). With further developing, the amnion begins to close gradually and to shift down below cardiac region (Fig. 1B). At 52 h, the heart looping is obvious, venous sinus and atrium appears in dorsal area and the ventricle and arterial conus appear in ventral area. Meanwhile, amniotic fold appears at the position that is ½ of the distance from heart to omphalomesenteric artery (Fig. 1C). At 54 h, amniotic fold shifts down to the position that is 1/3 of the distance from heart to omphalomesenteric artery (Fig. 1D). Then, the amniotic fold shifts down continually to the position above the omphalomesenteric artery (Fig. 1E). At 56 h, the upper limb buds appear, the amniotic fold heals near the omphalomesenteric artery. The heart becomes more obvious and larger compared to earlier stages. Once the cardiac tube closes completely, the venous sinus acts as the pacemaker and the heart begins to beat (Fig. 1F). At 65 h, the whole embryo bends further and the heart beats more vigorously (Fig. 1G). At about 75 h, the amniotic folds merges to an aperture, the truck crooks more, the atrium expands to the left (the back of embryo) and will be separated into right and left atriums soon. The venous sinus, which will develop into the right atrium, is located under the right side of atrium. The development of artery cone continues, which will divide into the main artery (Fig. 1H).

The Relation Between Different Embryo Developmental Stages and the Heart Morphological Characteristics

The cell movement at the beginning of the mesoderm formation forms a tiny-white trace at the caudal end of blastoderm at the beginning of embryo development. It is about 0.3-0.5 mm and is the earliest evidence of plumular axis. At 20 h, the primitive streak forms finally, which is 2.00 mm. At about 22 h head fold appears (Fig. 2A). At stage 24 h, the neural fold is not coalesced and the whole neural groove keeps open and 4 individual somites appear. In the mesoderm, a pair of incrassation regions appears, which will be a primitive heart forming region (Fig. 2B). At 30 h, the neural fold is coalesced. Both sides of endocardium get close to each other gradually and form the primitive cardiac

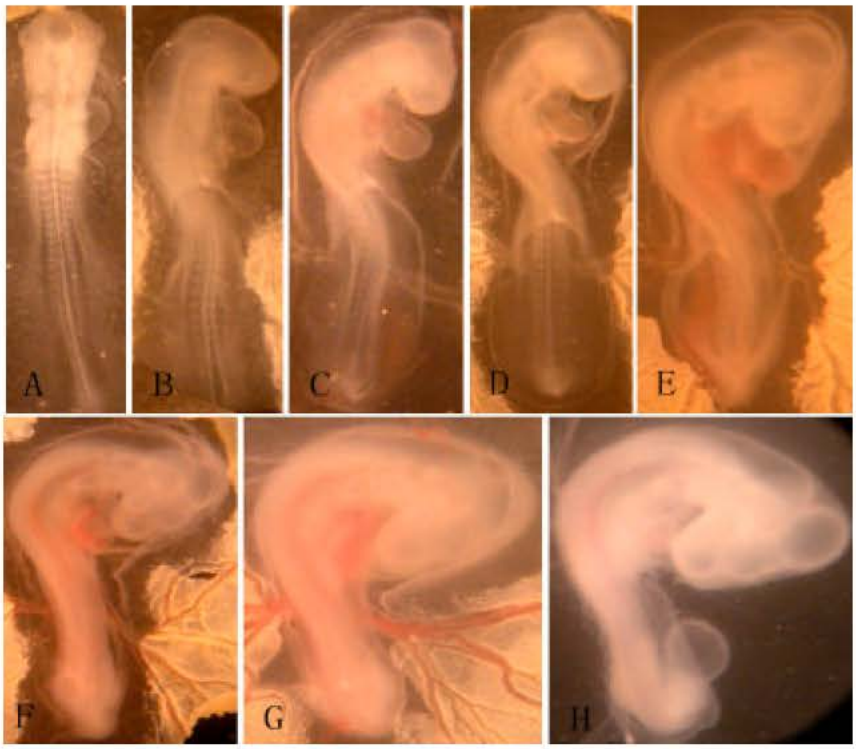

Fig. 1: The relation between the change of amniotic fold and cardiac development at different stages

tube (Fig. 2C). At 34 h, the cardiac tube starts to bend as U shape. Meanwhile, 3 primitive brain vesicles can be observed and a pair of optic vesicles appear in the front of brain (Fig. 2D). At 38 h, the embryo bends to the right side and the three primitive brain vesicles start to divide into five brain districts, endbrain, interbrain, midbrain, hindbrain and myelencephalon (Fig. 2E). At 48 h, amniotic edge coalesces to the edge of anterior intestine portal; heart loops and folds; ventricle, atrium, artery sinus, artery conus and the aorta arch III can be observed clearly (Fig. 2F). At 65 h, amniotic edge coalesces close to the artery of the omphalomesenteric artery; the ventricle and artery conus moves to the front (abdomen) and the atrium and artery sinus fold to the rear (back) (Fig. 2G). At 75 h, amniotic edge coalesces to the tail fold, forminga small aperture and the atrium expands. At 85 h, amnion edge coalesces completely; the heart tip appears; the atrium expands to the right and although no obvious demarcation line, the contraction area appears, which can be distinguished from ventricle; in this period, ventricle trabecula appears. After 100 h or later, the allantois becomes bigger; the heart with 4 chambers basically forms and the cardiac development is completed in essence (Fig. 2H).

Assuring accuracy and repeatability in the analysis is not only the precondition, but also the key point of the successful proteomics study. The two traditional standards, Hamburger-Hamilton (HH) and Witschi stages (Hamburger *et al*., 1951; Harh *et al*., 1975), are not suitable for the sample preparation for proteomic study because of the following reasons. Firstly, the dyeing will possibly influence the purity of proteins and thus will influence the results of the two-dimensional electrophoresis; secondly, the proteins are very easy to degrade in the course of dyeing and the only way to keep their integrality is to stripped the heart quickly and to preserved samples under -80°C; thirdly, the external development results in the individual difference of chick embryos, even if they are incubated for a same period under same condition. In order to set up a practical method to overcome these shortcomings, we dissected 2,000 chick embryos, separated and got about 1,500 of hearts and observed the morphologic characteristics of chick embryo in each stage.

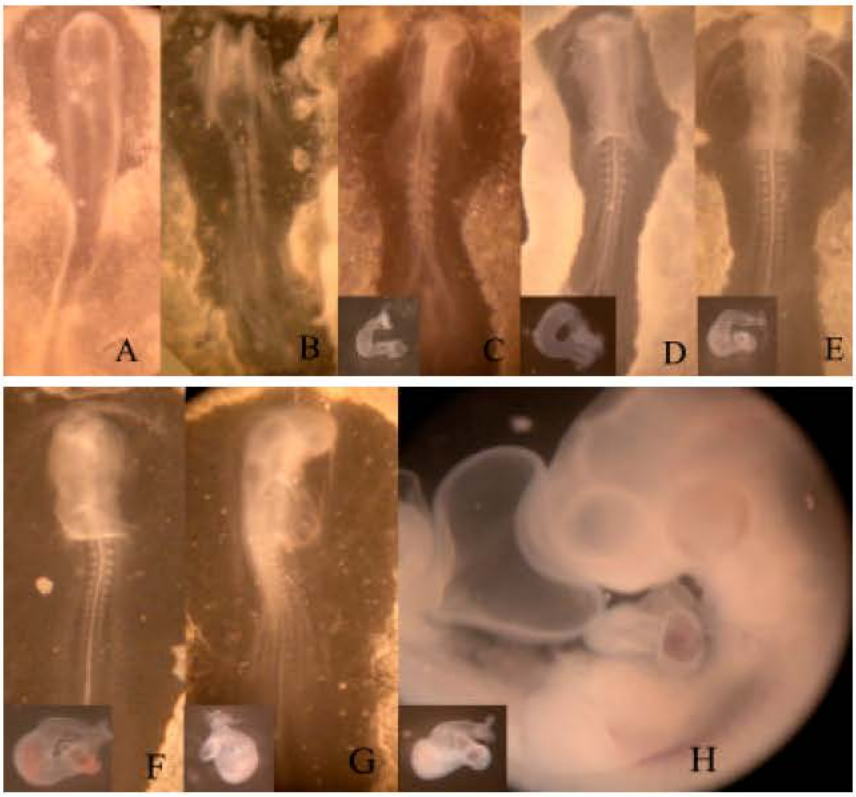

Fig. 2: The morphological characters of chick embryo heart at different stages. The small image on the left corner is the heart of the corresponding stage. A. At 22 h, the head fold appears. B. At 24 h, the neural fold keeps open. C. At 30 h, The neural fold closes and the primitive cardiac tube forms. D. At 34 h, the cardiac tube curves as U shape. E. Developmental stage 38 h. F. At 48 h, the heart loops and folds; ventricle, atrium, artery sinus, artery conus and the arch of aorta III can be observed clearly. G. At 65 h, the left and right atriums and artery sinus are folded to the back. H. After 100 h, the allantois becomes bigger; the heart with 4 chambers basically forms and the cardiac development is almost completed

We specially paid attention on the embryos of the stages between 48 to 100 h, because this is the important morphagenetic period of heart, during which a number of critical events of heart development happen (Garcia-Martinez *et al*., 1993). When the embryo developments to 48 h, the cardiac tube loops; to 56 h, the cardiac tube merges totally and begins to beat; to 65 h, heart looping shows its maximal curve; to 75 h, the atrium expands towards right and then the ventricle trabecula appears; after 100 h, atrioventricular septum forms basically, thus the typical heart with four distinct characteristic chambers comes into being (Martinsen, 2005).

The new method that we developed offers a work platform for the proteomics research on chick cardiac development. It remedies the insufficient of traditional standards, such as HH and Witschi, about the division of chick embryo development, confirms and subdivides the relation between the subtle events during chick heart development and relevant morphology characteristics, such as the folding and shifting of amnion and solves the problem that the external development is too inconsistent to be used to confirm the developmental stage. This standard can ensure a definite developmental stage accurately and quickly, offer a more scientific and more reliable basis for the collection of specific tissue at certain stage and assure the accuracy and repeatability of the process.

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