SHORT COMMUNICATION

Correlation of external ear auricle formation with staging of human embryos

Ozeki

Running title: Auricles of human embryos

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ABSTRACT

The formation of auricles in human embryos was evaluated between Carnegie stage (CS)19 and CS23, and the findings were correlated across the stages. The auricle was categorized into 11 steps according to Streeter's criteria with modifications. Mesenchyme cell condensation was observed at Step 7, and two layers of cartilage consisting of the auricle were recognized at Step11. The representative steps at each CS shifted from Step 3 to Step11 during CS16 and CS23, although several steps overlapped between adjacent CSs. These results indicate that observations of the auricle between CS19 and CS23 may be utilized for determining embryo staging as convincing supportive evidence of external features reflecting the internal histological structure, although other findings should also be taken into account.

Key Words: external ear, human embryo, Carnegie stage, auricle

INTRODUCTION

The external ear primordium arises on the mandibular and hyoid arches. At this point, six hillocks of the branchial arch appear, which disappear by fusing to establish the primitive auricles (Streeter 1922; O'Rahilly 1984). Since the morphology of the auricle is very unique and easily observable externally, it has been incorporated into assessments of the developmental staging of human embryos such as the Carnegie stage (CS) system (O'Rahilly and Müller 1987). The CS system relies on detailed morphology of the auricle from CS16 to CS18. Indeed, O'Rahilly and Müller (1987) suggested that the findings of the auricles are one of the most prominent and reliable factors for determining and discriminating between these three stages.

Streeter (1922) classified the formation of the auricles into 15 categories (A–O) during embryonic development (5–33 mm in crown-rump length). Based on the CS criteria (O'Rahilly and Müller 1987), categories C and D correspond to auricular formation at CS16, category E corresponds to that at CS17, and categories G, H, and I correspond to that at CS18 (Table 1, Figure 1). Although Streeter's (1922) categorization was not adapted to the CS system after CS19, six additional categories (J–O) were nonetheless recognized that approximately correspond to these periods.

Only two ambiguous descriptions have been recognized (O'Rahilly and Müller 1987): "the coalescence of parts in the pharyngeal region has altered the appearance of the auricle, and the hillocks are less conspicuous" (p. 238) at CS19 and "the formation of the auricle has progressed noticeably: the tragus and antitragus especially are assuming a more definite form" (p. 259) at CS22.

However, as O'Rahilly and Müller (1987) themselves pointed out, these descriptions are somewhat limited at the stage between CS19 and CS23 with respect to both external and internal findings. In fact, there are only 27 pages dedicated to the description of embryos between CS19 and CS23 in their book, whereas 79 pages are focused on the description of CS14 to CS18. Therefore, accumulation of new information on the external features corresponding to each stage may be useful to improve the accuracy of staging as well as to improve the precision of studies conducted during these periods. Nevertheless, it is necessary to take into account the developmental status of various internal structures in order to precisely assign the stage of a specimen after CS19. Considering this background, the aim of the present study was to observe the formation of auricles between CS19 and CS23, and provide quantitative data on the correlation between auricle formations and CSs.

MATERIALS AND METHODS

For external observations, 340 human embryo samples between CS16 and CS18, as well as 498 samples between CS19 and CS23 were selected from the Kyoto Collection (Nishimura et al. 1968; Shiota et al. 2007; Yamada et al. 2010). None of these samples exhibited overt damage or anomalies. The morphology of the auricle was observed from bilateral views.

The auricle was categorized into 11 steps according to the observations of Streeter (1922) with minor modifications (Table 1, Figure 1). In brief, five categories were omitted or combined from Streeter's 16 categories (A–O). Category A included only the samples before CS16. Category F was omitted because it was originally described as an exceptional, abnormal case. Category M was combined with category L, which was originally regarded as a variation of category L. Categories L and O were combined because the discrimination between these categories was not apparent in the original study, according to the following description "the transition from Category-N to -O brings us to a condition that may be regarded as the definitive auricle" (p. 130). When the

steps differed between the left and right auricles in the embryo, the step at a more progressed stage was defined as the step of the given sample. Each auricle was blindly categorized twice by one author (M.O), and confirmed by another author (T.T.).

For histological observations, 33 organs from 17 embryo samples between Step 7 and Step 11 were used from the Kyoto Collection. Histological serial sections stained with hematoxylin and eosin were observed microscopically by two authors independently (M.O. and T.T.). The ethics committee of the Kyoto University Graduate School and Faculty of Medicine approved this study (E986).

RESULTS AND DISCUSSION

Vague condensation of the mesenchyme cells as pre-cartilage was recognized around the external auditory meatus (EAM) at Step 7 (Figure 2). The meatal plug was recognized on the bottom of the EAM at Step 7. The distinct border was first recognized on the tip of the top of the raised margin (▲ in Step 8, Figure 2). Such condensation of the mesenchyme cells was recognizable from one of 4 samples at Step 7 and in all samples at Step 8 (Table 2). The centers of condensation increased in number as the steps proceeded (Steps 8-10). The hillocks were recognized as small

bulges rising into the EAM. Histologically, the cell density increased, but the border was not distinct. The border of the pre-cartilage became distinct on the side further from the EAM but not on the side closer to the EAM (step 9 in Figure 2).

A distinction between Step 10 and Step 11 was also revealed with the histological observation. The cartilage was clearly recognizable in 5 of 6 samples at Step 11 (Figure 2). The arrangements of the cells in the cartilage differed between the side closer to and that further from the EAM: the inner cells run horizontally while the outer cells run vertically. The extracellular matrix was scant compared with other cartilage observed in the same Step 11 samples, such as Meckel's cartilage and the otic vesicles. The epithelium of the external auditory canal contained 1–2 columnar cells until Step 7 (Table 2). The epithelium became stratified with 3–4 layers of cells in one of 4 samples at Step 7 and in all samples at Step 8. The stratified epithelium had over 4 layers at Steps 10 and 11. The histological observations indicate that the histological changes during development may contribute to and influence the appearance of the auricle at each step of its formation.

The representative steps to which most samples belonged at each CS shifted from Step 3 to Step 11 progressing from CS16 to CS23 (Table 3). Thus, the

representative steps at each CS were different, except between CS22 and CS23 (i.e., Step 11). The auricles of the samples ranged broadly between 4 and 5 steps between CS16 and CS18. On the other hand, the auricles of the samples ranged across less than 4 steps between CS20 and CS23; namely, 3 steps at CS20 (Steps 8–10) and CS21 (Steps 9–11), 2 steps at CS22 (Steps 10 and 11), and one step at CS23 (Step 11).

Overall, the range of steps to which the samples belonged overlapped between adjacent CSs. This means that the determination of the CS does not depend on the morphology of the auricle alone, but rather multiple findings of both the external and internal morphology are required for appropriate staging. This was the case even between CS16 and CS18, at which the findings of the auricle are considered to be the most reliable information for appropriate staging (O'Rahilly and Müller 1987). However, the CS can never be determined based on one absolute and dominant finding but instead requires accumulated information from several observations as a whole. In addition to the presence of auricular hillocks, the findings of limb buds, posture of the body, and facial features (nasal tip and wing, eye lids, etc.) are included as crucial findings for determining the stages between CS16 and CS18.

As for the later stages after CS19, detailed descriptions of external and internal findings are still in progress. External findings for discriminating between CS19 and CS23 were limited to the limb bud, posture of the limb, trunk, and head, eyelids, and the superficial vascular plexus of the head. The descriptions of these features are vague and/or do not always cover all stages between CS19 and CS23. For example, the descriptions of the toe ray are the most detailed at CS19, as toe rays are prominent but inter-digital notches have not yet appeared at that stage. However, this feature cannot be used in later stages.

The present study provides information on the differentiation of the auricles in a step-by-step manner, and demonstrates the correlation of these steps with CSs. The important finding is that auricle formation may be accurately represented by the histological features during development. These results indicate that observations of the auricle at these developmental periods may be utilized to determining staging, providing convincing supportive evidence of external embryonic features reflecting the internal histological structure, although other findings should also be taken into account simultaneously. These data may also be valuable for fetal three-dimensional sonographic studies, where accurate depiction of the fetal ear is important given that

ear anomalies can be associated with complex syndromes (Merz et al, 1997, Shih et al, 1998).

Acknowledgements

This study was supported by Grant Nos. 25461642, 24119002, 26220004, 15H01119, 15K08134, 15H05270, 15H01121, and 15K15014 from the Japan Society for the Promotion of Science.

Disclosures

None

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Table 1. Steps of auricular formation

Step	Main Feature
A. The appearance and development of branchial hillocks	
1	Two opaque elevations corresponding to hillocks 4 and 5 on the dorsal segment of the hyoid bar. An opaque thickening representing the first appearance of hillock 6 on the ventral segment of the hyoid bar. [B]
2	The three hyoid hillocks are clearly indicated. Hillock 1 is recognized on the ventral segment of the mandibular bar. [C, CS16]
3	Hillocks 4 and 5 are sharply rounded and have reached their maximum development. Hillock 6 becomes subdivided. The dorsal part of the mandibular bar shows hillocks 2 and 3. The hyoid cleft is widened to form a definite fossa. [D, CS16]
4	A total of six hillocks appear over the hyoid bar (hillocks 4 to 6) and the mandibular bar (hillocks 1, 2, 3), at their maximum development. [E, CS17]
B. The disappearance of branchial hillocks and formation of the primitive auricle	
5	Hillock 3 disappears, and hillock 2 is crowded to a more ventral point, which is preliminary to the formation of the Cms helicis. The fossa angularis forms a rather roomy quadrilateral depression whose floor bulges out slightly, [G, CS18]
6	Hillocks 1, 2, 6, and 6' are still clearly defined. Hillock 5 is becoming less distinct. Hillocks 3 and 4 can scarcely be outlined, which forms the rounded contour of the upper end of the fossa angularis. The depth of the fossa has increased. [H, CS18]
7	Hillocks 1 and 2 are still quite definite, while the last traces of hillocks 5 and 6 are to be seen. The raised margin of the fossa angularis begins to take the form of definitive parts of the auricle such as the Cms helicis (around hillock 3) and helix (around hillock 4). [I, CS18]
C. Establishment of the primitive auricle	
8	The last vestige of hillock 5 has disappeared. The fold of the helix appears to be more pronounced. [J]
9	The borders of the fossa angularis make up the sloping surface of the crus helicis and the primitive ear-fold or scapha-helix. Hillocks 1, 2, and 6' can still be recognized as the remnants. [K]

10	Hillocks 1 and 6' remain. The crus helicis is distinct and the primitive ear-fold is prominent. There is the early form of the concha, divided by the crus into an upper and a lower half.[L,M]
11	Transition to a stage that may be regarded as the definitive auricle, namely the tragus, antitragus, anthelix, scapha-helix, and, distinctly separate from the latter, the crus helicis is recognized.[N,O]

Letters in brackets indicate the categories that correspond to Streeter's (1922) study and the Carnegie staging of O'Rahilly and Müller (1987)

Table 2. Histological observation of the auricles

		Steps of auricular formation						
		5	**6**	**7**	**8**	**9**	**10**	**11**
Articular cartilage	(+)							5
	(+/-)			1	3	2	11	1
	(-)	3	3	4				
Epithelium of external	>4 layers						5	6
auditory canal	3-4			1	3	2	6	
	1-2	(3)*	(3)*	4				
Total	(n)	3	3	5	3	2	11	6

*; auditory canal is not differentiated from the outer skin.

(-); not recognizable

(+/-); recognizable as vague condensation of the mesenchymal cells

(+); distinct as the perichondrium border of cartilage

Table 3. Distribution of auricle formation according to Carnegie stage (CS).

CS	A. Development of branchial hillocks				B. Disappearance of branchial hillocks			C. Establishment of the primitive auricle				
	STEP 1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**Total**
16	12 (11.3)	33 (31.1)	52 (49.1)	9 (8.5)								106
17		2 (1.7)	25 (20.7)	93 (76.9)	1 (0.8)							121
18				8 (7.1)	44 (38.9)	41 (36.3)	16 (14.2)	4 (3.5)				113
19						4 (2.9)	28 (20.1)	96 (69.1)	11 (7.9)			189
20								19 (14.0)	73 (35.7)	44 (32.4)		136
21									20 (16.8)	96 (80.7)	3 (2.5)	119
22										12 (21.8)	43 (78.2)	55
23											49 (100)	49

Numbers in parentheses shows the percentage of each Carnegie stage

Figure legends

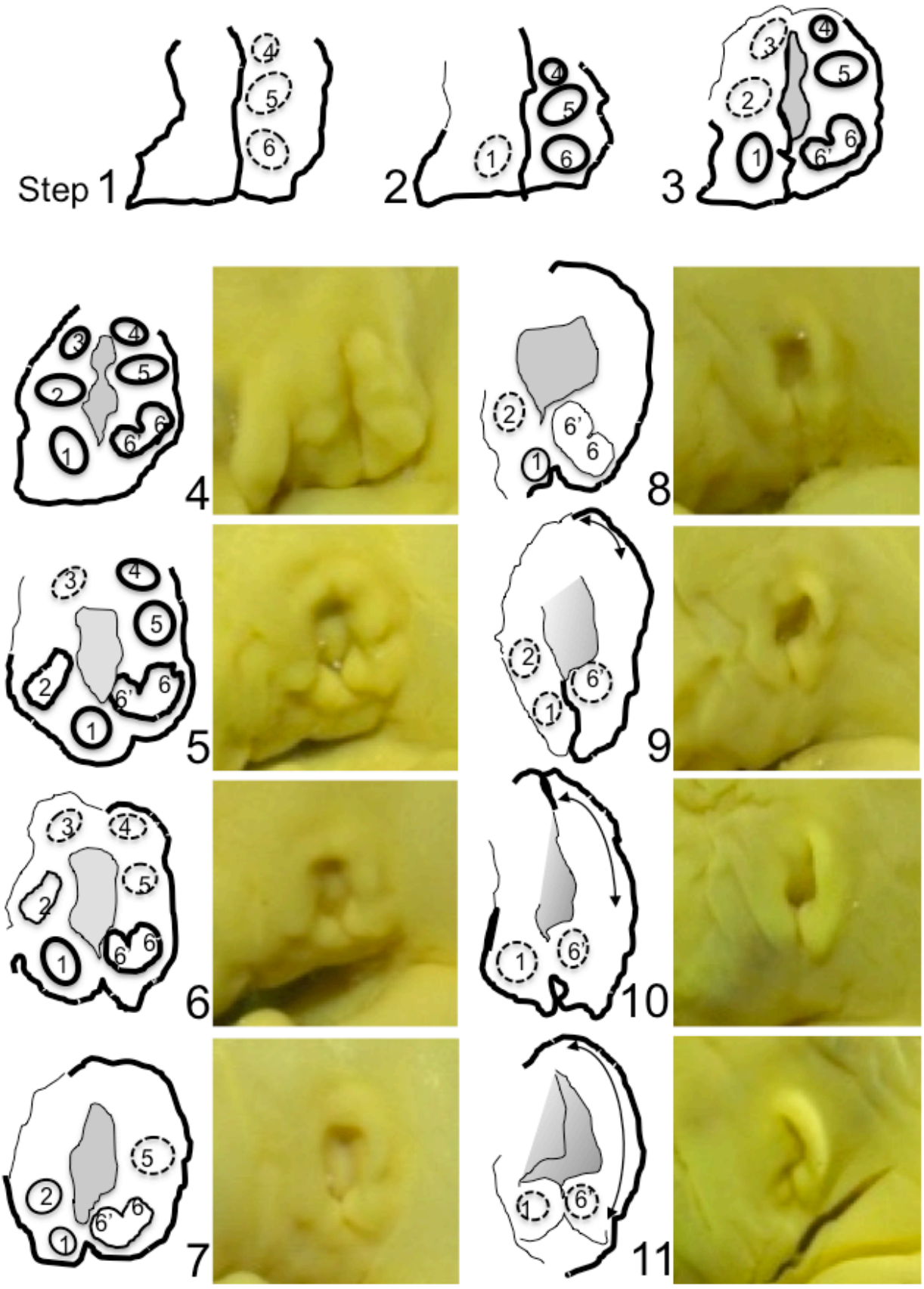
Figure 1. Representative findings of the auricles for all 11 steps

[Steps 1–4] Formation of the branchial hillocks until their maximum development; [Steps 5–7] Disappearance of the branchial hillocks; [Steps 8–11] Establishment of the primitive auricle.

The illustration was drawn with reference to Figure 5 and the associated descriptions found in Streeter (1922).

Figure 2. Histology of the auricles with hematoxylin and eosin staining from Steps 7 to 11

am; external auditory meatus, h; auricular hillocks, p; auditory pluq, ph; pharynx, m; Meckel's cartilage, 2; cartilage of the 2nd arch, at; auditory tube, oc; otic capsule


Step 1
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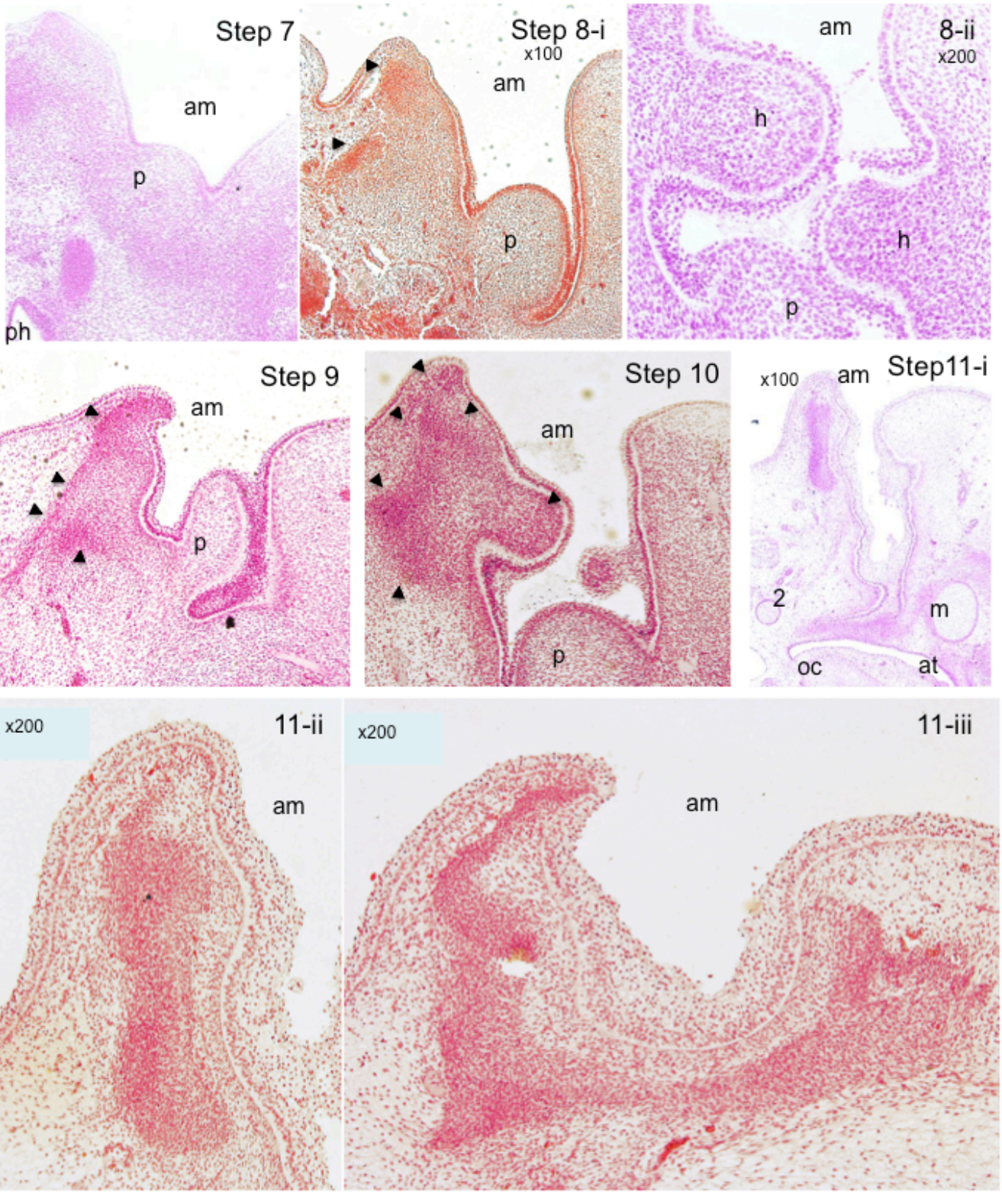
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