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Origin and development of the pronephros in the chick embryo

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Abstract

The process by which the pronephros develops was morphologically examined in chick embryos from Hamburger–Hamilton stage (ST) 8+ to ST34. The intermediate mesoderm, from which the pronephros arises, was first seen as a faint ridge of undifferentiated mesoderm between the segmental plate and lateral plate at ST8+. It formed a cell cord at the level of the 6th to the presumptive 13th somites at ST9 to ST10. This cell cord then separated into dorsal and ventral parts, the former becoming the nephric duct and the latter the tubules by ST14. The primordia of the external glomeruli (PEGs) appeared at ST15 through some epithelial cells protruding in the nephrostome (the opening of the nephric tubule into the body cavity). PEGs formed gradually in the caudal direction until ST18, while the pronephric tubules and PEGs in cranial locations disappeared. At this stage, only a few PEGs remained at the level of the 13th and 14th somites and these developed from ST23 to ST29 to become ultrastructurally similar to the glomeruli of the functional kidney. From these observations in the avian pronephros, we infer that the pronephric duct and tubules both form from a cell cord in the intermediate mesoderm and at the same time, but later develop differently.

Key words development; external glomerulus; kidney; mesonephros; nephrostome.

Introduction

It is well known that during the development of the kidney in the avian embryo, the pronephros, mesonephros and metanephros appear in sequence in a manner similar to that seen in mammalian and reptilian embryos (Felix, 1906; Goodrich, 1958; Arey, 1965; Romer & Parsons, 1977). Some descriptions of the development of the pronephros in the avian embryo have been made over the years on the basis of observations of paraffin sections (Gasser, 1877; Sedgwick, 1881; Felix, 1906; Abdel-Malek, 1950; Davies, 1950; Hamilton, 1952). With regard to the initial development of the pronephros, however, two incompatible theories exist: (1) nephric (pronephric or Wolffian) tubules initially arise from the intermediate mesoderm (segment stalk), then fuse to form the nephric (pronephric or Wolffian) duct (Felix, 1906; Waddington, 1938; Abdel-Malek, 1950; Hamilton, 1952; Romanoff, 1960; Balinsky, 1981) or (2) the nephric duct arises first and the nephric tubules appear later (Gasser, 1877; Sedgwick, 1881; Davies, 1950). The pronephros of the avian embryo has attracted little recent interest because it was considered to be a rudimentary organ that disappears without ever functioning, in contrast to the situation in fish (Holmgren, 1950; Euler & Fänge, 1961; Ellis & Youson, 1989) and amphibians (Fraser, 1950; Fox, 1963; Jaffee, 1963; Christensen, 1964).

In the last two decades, the nephric duct has been investigated in detail using the electron microscope, both with regard to its early development and its posterior extension (Jacob et al. 1986, 1991, 1992; Jarzem & Meier, 1987; Bellairs et al. 1995). These observations suggested that, in avian embryos, the nephric duct arises as a protrusion of cells from the intermediate mesoderm between the paraxial mesoderm and the lateral plate at a time before the pronephric tubules appear. Although some authors studying paraffin sections have concluded that, in avian embryos, the

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pronephros is temporary during development and functionless – because the nephric tubules and external glomeruli (EGs) of the pronephros are rudimentary and degenerate at early stages (Sedgwick, 1881; Felix, 1906; Abdel-Malek, 1950; Davies, 1950; Hamilton, 1952) – others have proposed that the pronephros may perform particular functions (Needham, 1931; Waddington, 1938; Jacob et al. 1977). For example, Jacob et al. (1977), who observed the EGs in the chick embryo at the 5-day stage by scanning and transmission electron microscopy (SEM and TEM), confirmed that EGs consisted of epithelial cells with foot processes, a thin basement membrane and a fenestrated endothelium, and suggested that the pronephros in the avian embryo may be functional.

In avian embryos, the pronephros consists of the pronephric duct and tubules together with EGs projecting into the body cavity, as seen in both fish (Ellis & Youson, 1989) and amphibians (Fox, 1963; Christensen, 1964). In addition, there is an intermediate zone in which EGs and internal glomeruli (IGs) often coexist or are fused in both avian embryos (Sedgwick, 1881; Davies, 1950) and reptilian embryos (Wiedersheim, 1890; de Walsche, 1929; Tribe & Fisk, 1941; Davies, 1950). From the existence of IGs, some investigators have deduced that the pronephros and mesonephros overlap each other; however, the boundary between the pronephros and mesonephros has been described in a variety of ways (Abdel-Malek, 1950; Davies, 1950; Hamilton, 1952) and the details remain unclear. As the functional significance of the pronephros is controversial, we have tried to clarify the situation and investigate in detail the origin and early development of EGs in the pronephros using a battery of methods that include SEM, TEM, vascular casts and light microscopy (LM) of serial, semithin Epon sections.

Materials and methods

Fertilized eggs of the domestic fowl, *Gallus gallus domesticus*, were supplied by the Poultry Station and the Livestock Center of Saitama Prefecture, and incubated at 38 °C. The chick embryos examined were from Hamburger–Hamilton stage (ST) 8+ to ST34 (Hamburger & Hamilton, 1951). Although Hamburger and Hamilton did not count the first somite after ST10 as it is rudimentary, we counted the first rudimentary somite at all observed stages, because a gap may otherwise arise in the counting of somite numbers. For example, although the embryo at ST12 has been said to have 16 somites, we call the last somite the 17th somite in the same embryo, because it really has 17 somites.

For SEM observations, the embryos were placed in plastic dishes and perfused via the heart with Locke solution (to wash out the blood) and then fixed with 2.5% glutaraldehyde mixed with 2% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.6. The ectoderm or endoderm with the splanchnic mesoderm was removed in the fixative with the aid of tungsten needles. After a brief rinse in 0.1 M cacodylate buffer, these specimens were refixed in buffered 1% OsO_4 for 1 or 2 h. They were then dehydrated through a graded ethanol series, critical-point dried in liquid CO_2, sputter-coated with platinum–palladium (15–20 nm in thickness) and examined using an SEM (S-4100, Hitachi, Japan).

For LM and TEM examinations, the initial fixation was carried out using the same procedure as that used for SEM. After a brief rinse in 0.1 M cacodylate buffer, embryos were refixed in buffered 1% OsO_4 for 1 or 2 h. They were then dehydrated through a graded ethanol series and embedded in Epon. The embryos were serially sectioned, either transversely or longitudinally, at 1-μm intervals through the pronephric region and stained with toluidine blue (for LM). The ultrathin sections for TEM were each mounted on a copper grid with a sufficiently large hole (*c.* 1×0.5 mm) to allow examination of the entire section. After staining with an aqueous solution of uranyl acetate and lead citrate, these sections were observed using a TEM (JEM-1010, JEOL, Japan).

For the preparation of vascular casts, the method of Hiruma & Hirakow (1995) was used. After the initial fixation had been carried out using the same procedure as that used for SEM, the embryos were injected manually with resin (Mercox, Dainippon Inc., Japan) via a syringe with a fine glass cannula. The injected specimens were then immersed in 15% KOH to remove tissues and washed with distilled water. After drying in air, the specimens were mounted on glass cover slips on aluminium stubs, sputter-coated with platinum–palladium (15–20 nm in thickness) and examined with an SEM microscope (S-4100 Hitachi, Japan).

Some embryos were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.6, the ectoderm removed in the fixative with the aid of tungsten needles and the embryos stained with haematoxylin and eosin after a brief rinse. After dehydration through a

Table 1 Number and size of EGs (or PEGs) in developing chick embryos

Stage	Time of incubation	No. of embryos	No. of EGs (or PEGs)		Long diameter (μm)		
			Total	Mean*	Mean	Max.	Min.
St14		8	0	0	–	–	–
ST15	55–63 h	8	30	1.9 ± 1.7	31.2 ± 11.3	69.8	18.3
ST16	60–68 h	12	90	3.8 ± 1.9	32.3 ± 11.7	65.8	13.1
ST17	65–72 h	14	152	5.4 ± 1.9	31.9 ± 10.3	65.0	14.1
ST18	3 days	10	131	6.6 ± 2.0	36.9 ± 14.8	112.8	14.6
ST19, 20	3–3.5 days	9	88	4.9 ± 1.8	43.4 ± 23.1	157.7	15.8
ST21, 22	3.5 days	10	91	4.6 ± 2.1	55.1 ± 28.5	163.5	20.5
ST23, 24	4 days	14	110	3.9 ± 1.7	73.0 ± 39.1	208.9	22.5
ST25, 26	5 days	13	61	2.4 ± 1.2	80.5 ± 44.1	192.7	23.7
ST27–29	6 days	9	25	1.6 ± 1.0	97.8 ± 44.2	184.0	34.0
ST30, 31	7 days	10	16	0.8 ± 1.0	93.3 ± 29.3	135.3	50.0
ST34	8 days	9	13	0.7 ± 0.7	89.2 ± 26.3	125.5	33.8

*Number of EGs (or PEGs) was counted on each side in chick embryos. Means are shown ± SD.

graded series of ethanols, whole-mount embryos on glass plates were observed using LM.

The number of external glomeruli (EGs) or their primordia (PEGs) that projected into the body cavity was counted in a total of 126 embryos (eight or more embryos per stage) from ST15 to ST34. On photographs enlarged four-fold from SEM images of EGs (250× original magnification), the long diameters of 804 EGs or PEGs were measured using vernier calipers (Table 1). We classified structures as EGs or PEGs depending on whether their epithelial cells did or did not have foot processes (Fig. 1).

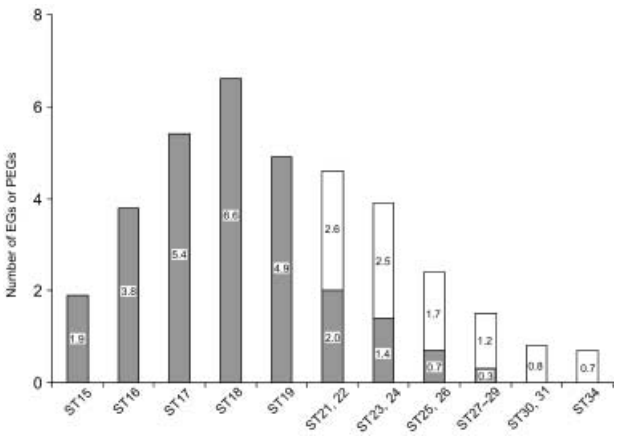

Fig. 1 Number of EGs (or PEGs) appearing in the body cavity on each side in chick embryos from ST15 to ST34. PEGs were considered to possess epithelial cells without foot processes (■), EGs to possess epithelial cells (podocytes) with foot processes (□). The latter appear at ST21, whereas the former are poorly developed and disappear at an early stage.

Results

Appearance of the pronephric rudiment

At ST8+ (five somites) LM observations of whole-mount embryos from which the ectoderm had been removed showed a deeply stained strand in the mesoderm lateral to the segmental plate on each side from the level of the presumptive region of the 6th to that of the 8th somite (Fig. 2A). At ST9 (seven somites), this strand became sharper in outline and could clearly be seen from the level of the 6th somite to a distance equivalent to about three somites beyond the last somite (Fig. 2B). In serial transverse Epon sections, the intermediate mesoderm between the segmental plate and lateral plate was distinguishable by a faint condensation of cells at ST8+ (Fig. 2C) and, by ST9, was slightly elevated toward the ectoderm (Fig. 2D). In SEM images of the dorsal surface of ectoderm-less embryos at ST9+ (eight somites), the intermediate mesoderm was distinguishable by shape and by arrangement of cells between the paraxial mesoderm and the lateral plate (Fig. 2E,F). At this stage, it displayed a slender, spindle-like shape and was elongated cranio-caudally, extending from the level of the 6th somite to that of the presumptive 11th somite. At ST10 (ten somites), the intermediate mesoderm formed a cell cord from the level of the 6th somite to the presumptive region of the 12th somite (Fig. 2G). This cell cord has been called the primordium of the pronephric duct by Jarzem & Meier (1987); and its constituent cells were orientated cranio-caudally (Fig. 2H). The caudal tip of this cord subsequently extended rapidly (by comparison with

Fig. 2 LM images of whole-mount preparations (A,B), SEM images of dorsal surfaces of chick embryos from which the ectoderm had been removed (E–I) and LM images of semithin Epon sections (C,D). (A) ST8+ (five somites). Arrowheads: an initial intermediate mesoderm from the level of the presumptive 6th somite (PS6) to about PS9. (B) ST9 (seven somites). Arrowheads: condensed region extending from the level of the 6th somite (S6) to about PS10. (C) Cross-section through PS7 at ST8+ (five somites). Arrowheads: initial intermediate mesoderm. (D) Cross-section through PS8 at ST9 (seven somites). The intermediate mesoderm is elevated slightly toward the ectoderm (arrowheads). (E) ST9+ (eight somites). The intermediate mesoderm is seen from a level slightly cranial to S6 to about the level of PS12 (enclosed by the broken white line). (F) Enlargement of the rectangle in E. Flat, polygonal cells with many filopodia connect loosely with each other. (G) ST10 (ten somites). A cell cord of intermediate mesoderm is arranged from the S6 level to PS14 (arrows). (H) Enlargement of the rectangle in G. The cell cord consists of cells lengthened in the cranio-caudal direction. (I) ST12+ (18 somites). The cranial part of the cell cord at the level cranial to S14 is covered by mesenchymal cells. The caudal part is lengthened caudally to a distance equivalent to about five somites beyond the last somite (arrow). Scale bars in A–E, G and I = 50 μm; F and H = 10 μm.

the rate of somite formation) and came to extend to a distance equivalent to five somites beyond the 18th somite at ST12+ (18 somites; Fig. 2I).

In serial Epon sections of embryos at these stages, the intermediate mesoderm consisted of a solid cell mass and was easily distinguishable from the paraxial mesoderm and lateral plate at ST9+ (Fig. 3A). At ST10+ (12 somites) and ST11– (13 somites), some differences in the arrangement of cells in the cell cord were noted between its dorsal and ventral parts at the level of the 6th to the presumptive 15th somite (Fig. 3C,D).

At the level of the 6th to 10th somites at ST11, the dorsal mass of the intermediate mesoderm was compactly arranged in a semicircular shape, whereas the ventral mass curved along the contour of the dorsal aorta (Fig. 3B). At the level of the 11th to the presumptive 14th somite, the dorsal mass was rough and spread laterally, whereas the ventral mass was similar to that in the cranial part (Fig. 3C). At a level caudal to the presumptive 15th somite, the dorsal mass had separated from the ventral mass and extended over both it and the lateral plate, whereas the ventral mass was solid and flat-plate-like (Fig. 3D). Observations on serial Epon sections confirmed that the isolated dorsal mass was continuous with the superficial part of the intermediate mesoderm lying cranial to it.

By contrast, the anterior part of the cell cord at the level of the 5th to 6th somites began to lose its cell density and to become indistinguishable from the surrounding mesenchyme. At ST12 (17 somites), the

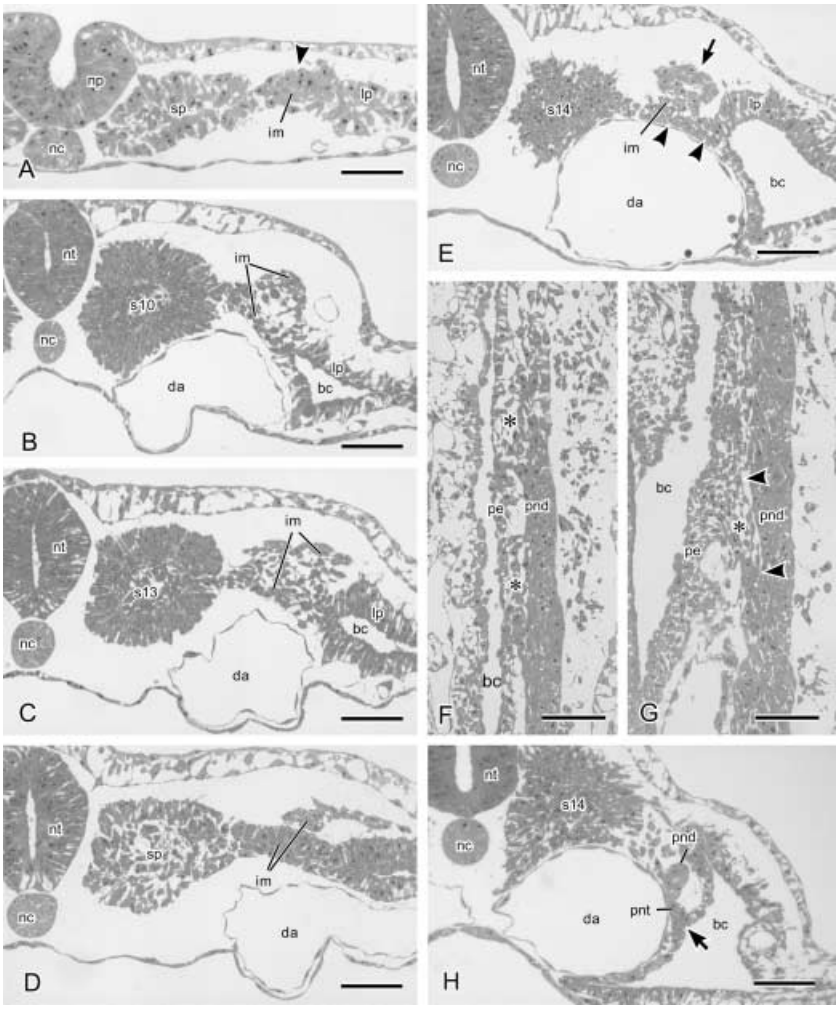

Fig. 3 LM images of Epon cross-sections (A–E, H) and longitudinal sections (F,G) of chick embryos. (A) ST9+ (eight somites) through the segmental plate at the PS9 level. The intermediate mesoderm is elevated slightly in the dorsal direction (arrowhead). (B–D) ST11 (13 somites), through S10, S13 and the segmental plate at the PS15 level, respectively. The cell arrangement in the intermediate mesoderm differs between the dorsal and ventral parts. (E) ST12 (17 somites), through S14. In the cell mass of the intermediate mesoderm, the dorsal part is a tubule-like structure (arrow) and the ventral part is arranged in two epithelial-like layers (arrowhead). (F) ST13– (19 somites), at the S9 to S11 level. Small cell cords (asterisks) are seen between the primordium of the nephric duct and the peritoneal epithelium. (G) ST13– (19 somites), at the S11 to S13 level. The cell cord (asterisk) connects obliquely between the peritoneal epithelium and the primordium of the nephric duct (arrowheads). (H) ST13 (20 somites), through S14. The nephric tubule joining the nephric duct opens into the body cavity (arrow). Scale bar = 50 μm.

superficial dorsal cell mass at the level of the 8th to 15th somites formed a tubular structure that was separate from the ventral cell mass (Fig. 3E). At this stage, the deep ventral cell mass was arranged in two epithelial-like layers and was continuous with the peritoneal epithelium (Fig. 3E), whereas the intermediate mesoderm at the level of the 7th somite became fragmented and rudimentary. At ST13– (19 somites), the dorsal cell mass of the intermediate mesoderm at the level of the 8th to 13th somites comprised an epithelial tube with a narrow, discontinuous lumen. Serial longitudinal Epon sections revealed that thin cell cords had formed between the dorsal cell mass and peritoneal epithelium at ST13– (19 somites) owing to the appearance of gaps (Fig. 3F,G). At the level of the 12th to 13th somites, thin cell cords running obliquely and cranio-caudally extended the peritoneal epithelium cranially to the point of connection with the dorsal cell mass (Fig. 3G).

At ST13 (20 somites), a continuous lumen arose in the dorsal cell mass of the intermediate mesoderm at the level of the 10th to about the 17th somite. As a consequence, the nephric duct was established and came to occupy a position lateral to the dorsal aorta (Fig. 3H). At the same time, the thin cell cords present between the nephric duct and peritoneal epithelium became a tubule that opened into the body cavity (Fig. 3H). The intermediate mesoderm positioned cranially to the 10th somite degenerated into a few vesicle-like remnants, some or all of which connected with the tubules opening into the body cavity. The nephric tubules at the level of the 10th to 12th somites were thinner and shorter than those at the level of the 13th and 14th somites.

Appearance of nephrostomes

SEM observation of the openings of the nephric tubules showed that nephrostomes could be recognized as

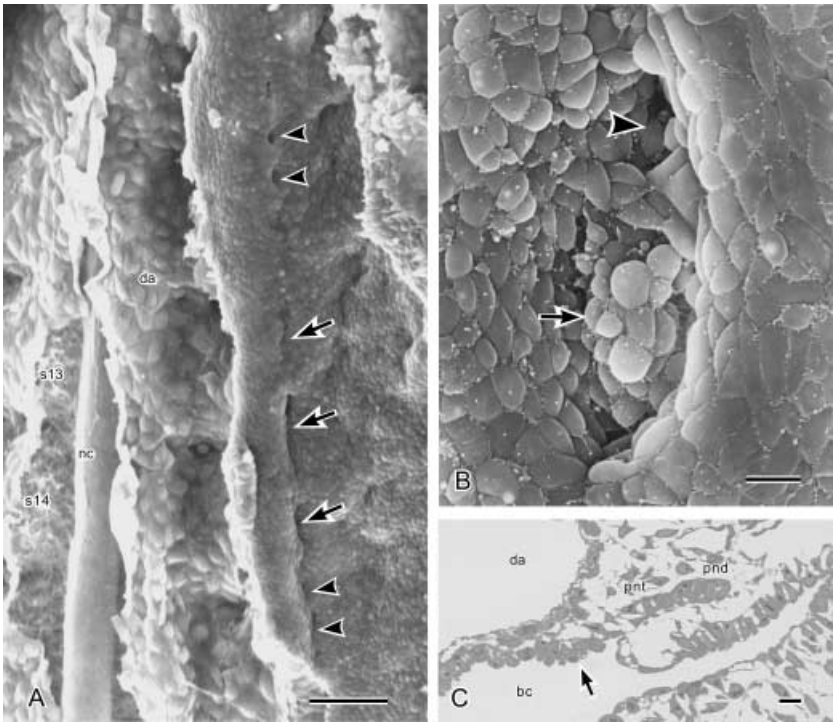

Fig. 4 SEM images (A,B) and LM image of Epon cross-section (C) of chick embryos. (A) ST13 (21 somites). Nephrostomes are recognized on the dorsal surface of the body cavity as small depressions (arrowheads) or depressions lengthened cranio-caudally (arrows). (B) ST15 (27 somites). A protrusion of cells is seen in the large nephrostome (arrow) at the S14 level but not in the small nephrostome (arrowhead). (C) ST15 (27 somites), through S14. The epithelium of the large nephrostome protrudes into the body cavity (arrow). The small cell cord is the primordium of the nephric tubule. Scale bar in A = 50 μm; B and C = 10 μm.

longitudinally arranged pits of various size at levels between the 8th and 17th somites (Fig. 4A). The nephrostomes lying at the level of the 13th to 14th somites were arranged lengthwise and were slit-like, whereas those cranial and caudal to them were round and small. At ST15 (27 somites), the nephrostomes were distributed caudally as far as the level of the 20th somite, and rounded protrusions of cells appeared in the medial walls of some nephrostomes at the level of the 8th to 14th somites on each side of the dorsal mesentery (Fig. 4B). Serial Epon sections taken at the same level at this stage revealed that the cells of these protrusions were the primordia of the external glomeruli (PEGs) and were continuous with both the epithelial cells of the peritoneum and the nephric tubules (Fig. 4C).

Boundary between the pronephros and mesonephros

By ST17, the PEGs existing in the nephrostomes cranial to the level of the 14th somite varied in size, whereas the nephrostomes caudal to the level of the 15th somite had no PEGs and were seen on the SEM images as pits in the wall of the body cavity (Fig. 5A). After ST18, the nephrostomes caudal to the level of the 15th somite became unclear or disappeared. In longitudinal serial Epon sections of embryos at ST17 and ST18, the nephric duct cranial to the level of the 14th somite consisted of columnar epithelium and was thin (about 25 μm in diameter with a minimal lumen), whereas at the level of the 15th and 16th somites it was 40–50 μm in diameter and had a obvious lumen (Fig. 5B). Moreover, at ST17 the primordia of the internal glomeruli (PIGs) appeared as protrusions of cells into the widened nephric tubules at the level of the 15th and 16th somites. The part of the nephric duct cranial to the level of the 14th somite was therefore termed the pronephric duct to distinguish it from the mesonephric duct, which was located caudal to the level of the 14th somite. The pronephric and mesonephric tubules were distinguished in the same way. In longitudinal serial Epon sections, the mesonephric tubules formed oblique links between the Wolffian duct caudally and the peritoneal epithelium, as did the pronephric tubules (Fig. 5C,D).

Growth of PEGs

The PEGs gradually increased in number as development progressed, reaching a maximum at ST18, when there were an average of 6.6 PEGs on each side of the embryo (Table 1 and Fig. 1). In SEM images of embryos at this stage, the PEGs varied in size, were arranged in a single row on each side at irregular intervals and were either spherical or oval (elongated cranio-caudally) (Fig. 6A). The surface of the cells comprising them

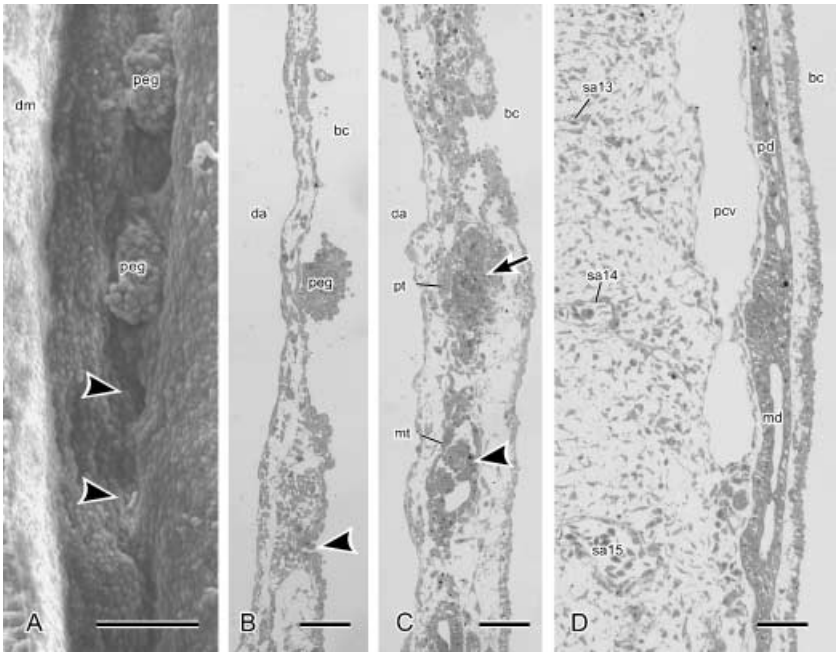

Fig. 5 SEM image (A) and LM images from Epon serial sections (B–D) of chick embryos at ST17. The longitudinal sections (B–D) are cut through the pronephros at almost the same level as A. (A) Ventral surface at the S13 to S16 level. PEGs and nephrostomes (arrowheads) are seen along the dorsal mesentery. (B) Section through the most caudal PEG, at the S14 level. A nephrostome of the mesonephric tubule opens into the body cavity (arrowhead). (C) Section 13 μm lateral to B. The PEG in B is seen continuously in the pronephric tubule (arrow). Caudal to it, a dilated mesonephric tubule with a cell mass inside it (arrowhead) is seen. (D) Section 56 μm lateral to C. The boundary between a slender pronephric duct and a thick mesonephric duct is present at a level between S14 and S15. Scale bar = 50 μm.

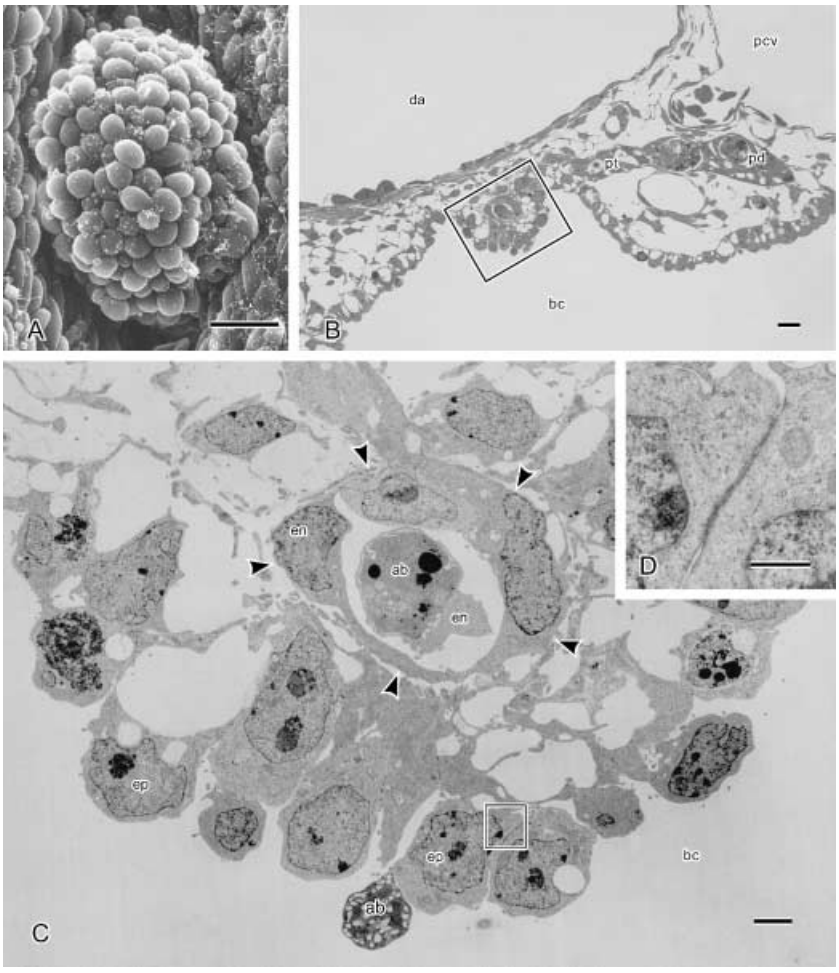

Fig. 6 SEM image (A), LM image in semithin Epon section (B) and TEM image (C,D) of PEGs in chick embryos at ST18. (A) A large PEG at the S12 level consists of hemispherical cells with small microvilli. (B) Cross-section at same level as A. The PEG (enclosed by rectangle) is adjacent to the dorsal aorta. A pronephric tubule extends from the nephrostome to the pronephric duct. (C) Enlargement of the area enclosed by the rectangle in B. The PEG consists of epithelial cells with irregular processes and a primordium of a blood vessel (arrowheads). Apoptotic bodies are seen in both epithelium and endothelium in the PEG. (D) Enlargement of the area enclosed by the rectangle in C. Epithelial cells connect tightly with each other via junctional complexes. Scale bar in A and B = 10 μm; C = 2 μm.

was hemispherical and each had a small number of microvilli (Fig. 6A). In serial Epon sections at the level of the 13th somite in an embryo at the same stage, the PEGs were seen to be positioned ventro-medial to the dorsal aorta, and the pronephric tubule was elongated to form a cord that connected the Wolffian duct to the peritoneal epithelium (Fig. 6B). Furthermore, TEM observation of such PEGs revealed that the superficial cells gave off irregular processes from their base toward the internal part of the PEG (Fig. 6C), connected with neighbouring cells by adherence junctions and had a thin basement membrane in the basal portion (Fig. 6D). The primordium of a blood vessel, with a lumen, was present close to the centre of this PEG (Fig. 6C). In serial Epon sections, most PEGs at this stage did not yet have blood vessels within them and only a few had connected to the dorsal aorta. Mesenchymal cells were scattered at various densities between the peritoneal epithelium and the primordium of the blood vessels and, moreover, apoptotic bodies were frequently noted in the PEG epithelium, the vascular wall and the mesenchyme (Fig. 6C). The degree of development of the PEGs containing the primordia of blood vessels varied and seemed to be independent of the level at which they were located.

Appearance of capillary tufts and podocytes in PEGs

As development proceeded, PEGs gradually decreased in number (Table 1) and differences in both size and shape occurred among them within a given embryo (Fig. 7A). At ST21, small PEGs (under 50 μm in diameter) remained unchanged in appearance and size (as compared with those at ST18) (Fig. 7B) but larger PEGs (more than 50 μm in diameter) had spindle-shaped epithelial cells with wide lateral processes that frequently had secondary processes (Fig. 7C). In serial Epon sections of large PEGs, the blood vessels inside, which had

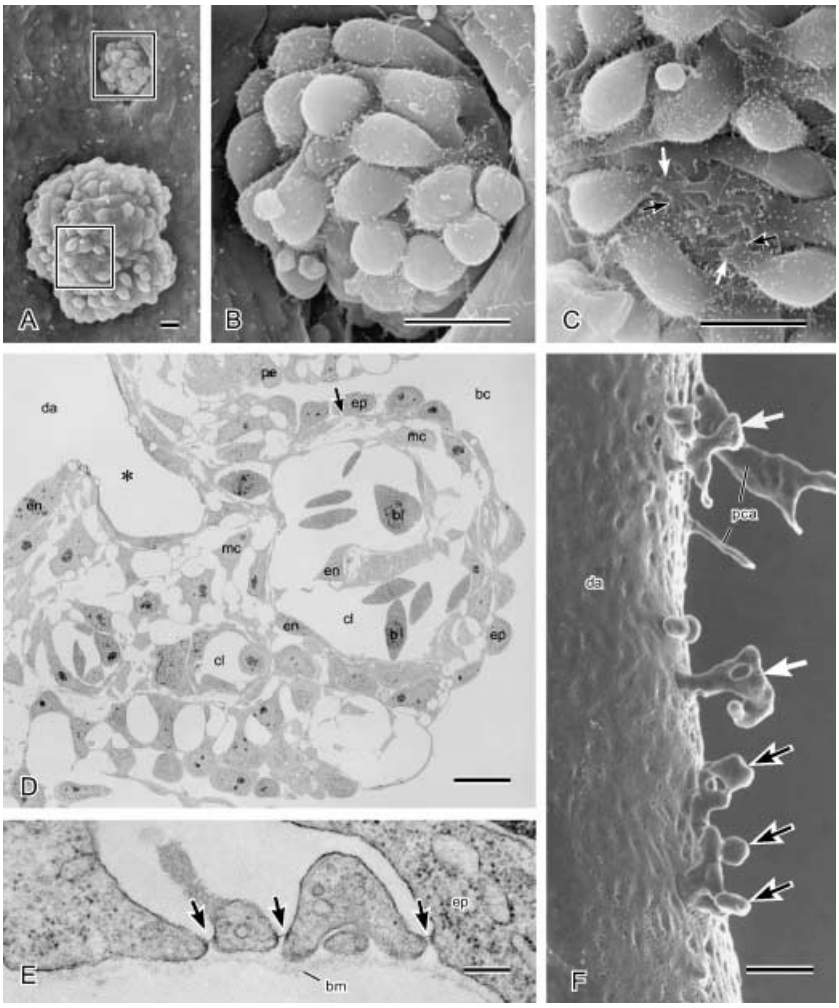

Fig. 7 SEM images (A–C), LM and TEM images (D,E) of a PEG, and an SEM image of a vascular cast (E) in chick embryos at ST21. (A) Two PEGs at the S10 and S11 levels differ in size. (B) Enlargement of the area enclosed by the upper rectangle in A. The small PEG consists of hemispherical cells and is not morphologically different from that seen at ST15 (Fig. 4B). (C) Enlargement of the area enclosed by the lower rectangle in A. The large PEG consists of spindle-shaped cells with long primary (white arrows) and short secondary (black-on-white arrows) processes. (D) Vascular tufts are developing in the large PEG. A branch from the dorsal aorta invades into the PEG. (E) High magnification of the area indicated by an arrow in D. Gap and slit membranes are recognized among the processes of the epithelial cells (arrows). A thin basement membrane is present beneath the epithelium. (F) Right-side view of the dorsal aorta and its branches at the S13 to S15 level. White arrows indicate the vascular tufts of the pronephric PEGs, whereas black-on-white arrows indicate those of the mesonephric PIGs. Scale bar in A–D = 10 μm; F = 50 μm; E = 200 nm.

a slightly complicated capillary loop, could be seen to connect to a branch (pronephric artery) of the dorsal aorta (Fig. 7D). In TEM micrographs, the epithelial cells of these well-developed PEGs could be seen to be hemispherical, to protrude into the body cavity in the centre, where the nucleus was situated, and to be thin at the cell periphery (Fig. 7D). Secondary and tertiary processes lay between these cell bodies and were frequently separated by slits that were spanned by a slit membrane, whereas a thin, discontinuous basement membrane was found beneath the epithelium (Fig. 7E). A variety of wide spaces was present between the epithelium of a PEG and the endothelium of the capillaries within it, and these spaces were occupied by one or two layers of mesenchymal cells arranged irregularly (Fig. 7D). In addition, SEM observations of vascular casts revealed vascular tufts within PEGs for the first time at ST19 (Fig. 7F), and we also noted that the PEGs of the pronephros were located slightly medial to the PIGs of the mesonephros. When we compared the location of the vascular tufts, those within the PEGs in the pronephros were further from the dorsal aorta than those within the PIGs in the mesonephros, and it was therefore easy to distinguish which vascular tufts belonged to the pronephros and which to the mesonephros (Fig. 7F).

Completion and degeneration of the EG

SEM observations revealed that, in embryos from ST23 to ST29, very large PEGs were present in very small numbers (Fig. 8A and Table 1). These PEGs, which were present at the level of the 13th to 15th somites at ST23, shifted caudally as development proceeded and began to be seen at the level of the 16th to 18th somites in embryos from ST27 to ST29. Their epithelial cells had well-developed secondary and tertiary processes (foot processes), which were interdigitated intricately with each other (Fig. 8B). We therefore referred to such PEGs as external glomeruli (EGs) at or after this stage. In serial Epon sections of an EG at the same stage, the vascular tufts inside were connected with each other, and IGs connecting with the EGs existed in the abdominal wall (Fig. 8C). SEM of vascular casts clearly showed that the EGs were connected to adjacent IGs by a small blood vessel (Fig. 8D,E). The capillary loops of the EGs

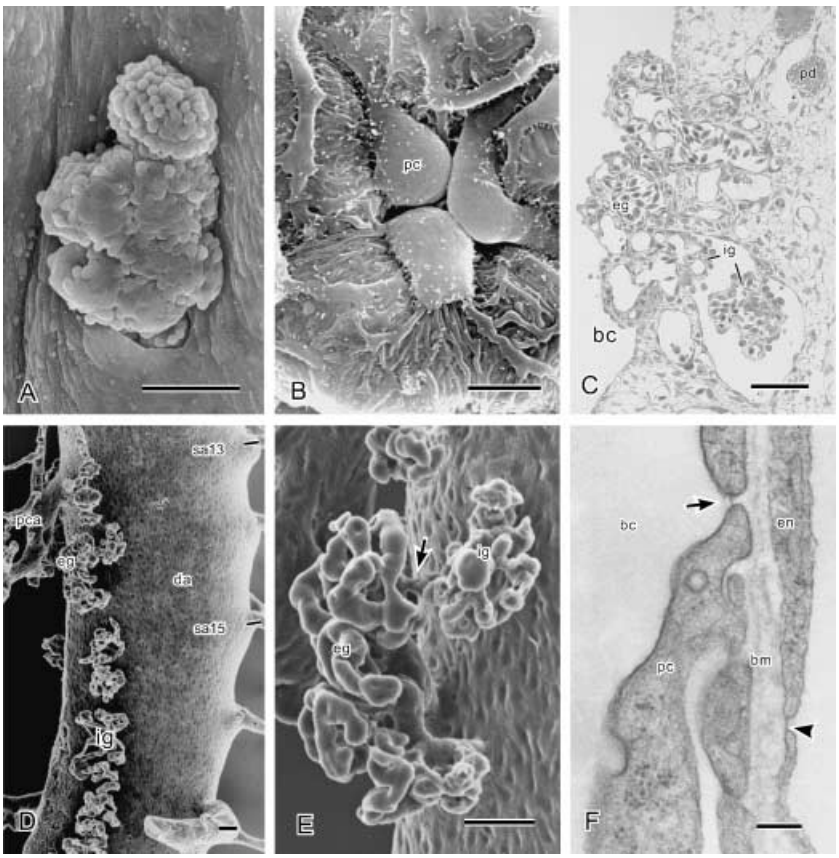

Fig. 8 SEM images (A,B), LM image (C), SEM images of a vascular cast (D,E) and TEM image (F) of a well-developed EG in chick embryos. (A) A large EG at the S15 level at ST26 is a conjugate of two or three EGs and is partly embedded in the peritoneal wall. (B) Enlargement of part of A. Podocytes with well-development foot processes interdigitate with each other on the EGs. (C) A longitudinal section of an EG similar to that in A. The EG and IG are supplied by a common capillary. (D) Left-side view of the dorsal aorta at the level of the 13–15th segmental arteries at ST24. The capillary loops of EGs in the pronephros are seen at the level of the 13–15th segmental arteries (sa13–sa15), while IGs in the mesonephros arise caudal to them. (E) Enlargement of part of D. The vascular tuft consists of a large EG and a small IG. (F) High magnification of the large EG. The wall of the EG consists of the foot processes of podocytes together with endothelial cells and a basement membrane between the two. A slit membrane between foot processes (arrow) and the thin diaphragm of an endothelial pore (arrowhead) are recognized. Scale bars in A and C–E = 50 μm; B = 5 μm; F = 200 nm.

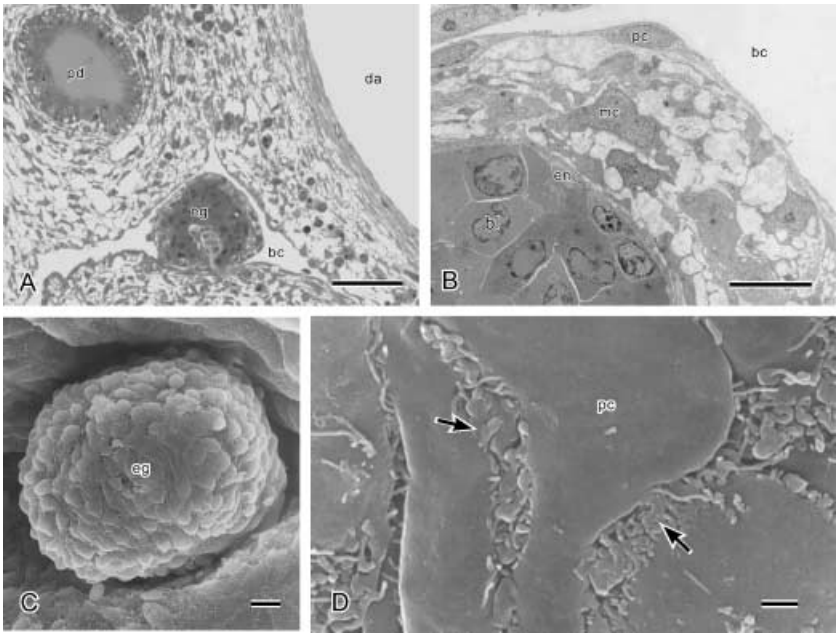

Fig. 9 LM image (A), TEM image (B) and SEM images (C,D) of a degenerating EG. (A) A relatively large EG in the body cavity at ST28. The blood vessel is filled with blood corpuscles. (B) Enlargement of part of A. The wall of the EG consists of mesenchymal cells and extracellular matrix. (C) An EG in the body cavity at ST34. The surface of the EG is covered with hemispherical cells. (D) Enlargement of part of C. The foot processes of the epithelial cells (podocytes) are comparatively short and rounded (arrows). Scale bars in A = 50 μm; B and C = 10 μm; D = 1 μm.

in the pronephros were seen at the level of the 13–15th segmental arteries, whereas the IGs in the mesonephros arose caudal to them (Fig. 8D).

TEM examination at high magnification revealed that the space between podocytes and the highly flattened endothelial cells was narrow and that only a thin and continuous basement membrane was present (Fig. 8F). Filtration slits with a thin membrane were present between the foot processes of the podocytes, and the endothelial cells were fenestrated by pores with a diaphragm. Such EGs reached maximum size at ST28, when their long diameter was on average 112.4 μm. From ST26 onwards, degeneration of the pronephros occurred, i.e. in serial Epon sections it was confirmed (i) that EGs did not communicate with vessels outside themselves and (ii) that the large EGs had no relation to the pronephric duct and were consequently isolated within the body cavity (Fig. 9A). Such EGs were filled with blood corpuscles, and their walls were thickened with a loose cell layer of mesenchymal cells and extracelluar matrix (Fig. 9B). Even at ST30 to ST34, some EGs remained on one or both sides of embryos but the podocytes of which they were composed were flat in shape and had short or degenerate foot processes (Fig. 9C,D). The epithelial cells of the EGs under about 50 μm in long diameter had no foot processes and were scarcely different in size or in appearance from those at the initial stage. They began to decrease in number at about ST19, spontaneously degenerating and disappearing by ST28 (Table 1 and Fig. 1).

Discussion

The development of the pronephros in the chick embryo, which was examined in this study, is summarized in diagrammatic form in Fig. 10. This illustrates the growth and degeneration of the nephric duct and EG as well as of the nephric tubules.

Appearance of the intermediate mesoderm

It has previously been reported that the intermediate mesoderm (intermediate cell mass) is initiated as a slight projection of the mesodermal region between the paraxial mesoderm and lateral plate in eight-somite chick embryos, with some differences in position at about the level of the 5th to 10th somites (Gasser, 1877; Sedgwick, 1881; Jacob et al. 1986). In the present study, the first appearance of the intermediate mesoderm was somewhat earlier than in the above reports, and at the level of the presumptive 6th to 8th somites in whole-mount preparations of the five-somite chick embryo (ST8+). In embryos with 6–10 somites, the region of the intermediate mesoderm apparently extended in the caudal direction to a distance equivalent to only about three somites beyond the last somite (Fig. 10).

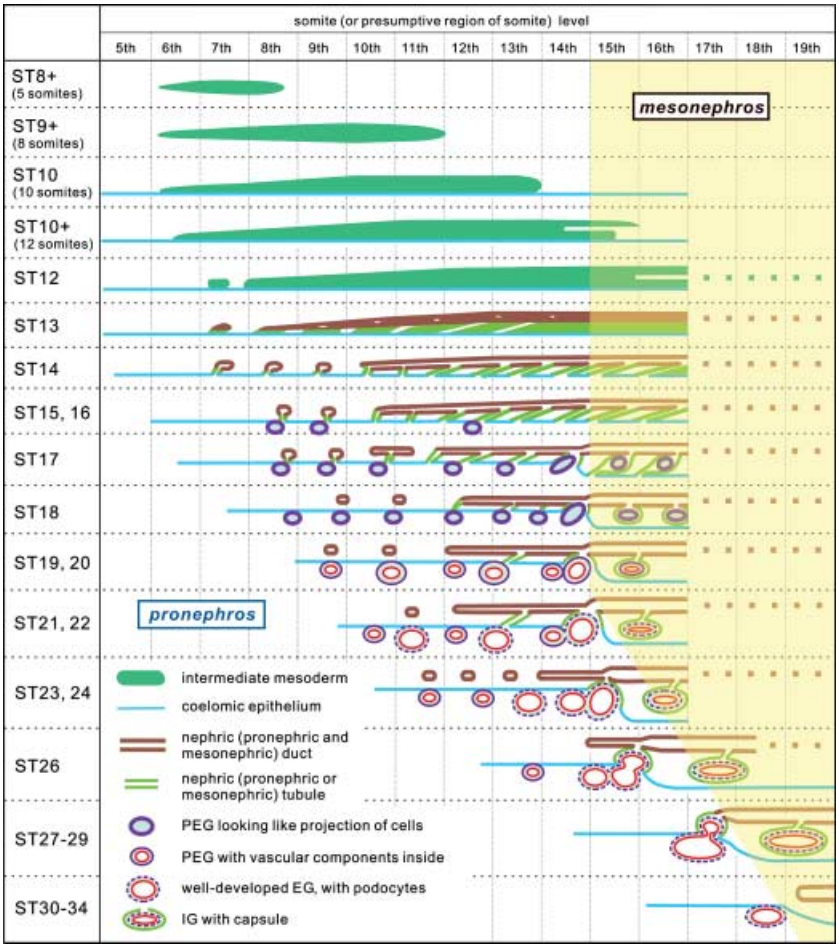

Fig. 10 Schematic colour diagram showing the process of development of the pronephros in chick embryos from ST8+ to ST34. The cream-coloured area shows the region of the mesonephros (which was omitted except for its most cranial portion).

Pronephric duct and tubules arise from the intermediate mesoderm

Jacob et al. (1979) and Jarzem & Meier (1987), using SEM, described the intermediate mesoderm as forming a cell cord extending from the level of the 6th to the presumptive 13th somite in the ten-somite chick embryo. These authors termed the cell cord a nephric (or pronephric) duct rudiment and emphasized that the nephric (or pronephric) duct did not arise from a fusion of pronephric tubules in chick embryos. Actually, more than a century ago Gasser (1877) and Sedgwick (1881) had already described a similar process in the formation of the Wolffian (nephric) duct and tubules in chick embryos. Their findings have not been widely recognized, with the theory put forward by Felix (1906) on the basis of phylogeny being generally used (Carlson, 1996; Bellairs & Osmond, 1998).

As regards the formation of the pronephric tubules, Jarzem & Meier (1987) suggested from their SEM findings that the pronephric duct has a different origin from the pronephric tubules in chick embryos and that the former may play an inductive role in the formation of the latter. Sedgwick (1881) noted that the Wolffian (nephric) duct and very short tubules appeared as a narrow groove in the peritoneal epithelium at the level of the 7th to 11th somites in chicks at 19- to 32-somite stages. However, there has been no detailed examination of the canalization of the pronephric tubules. According to our observations of serial Epon sections of embryos, at about ST12 the cell cord separates into dorsal and ventral cell masses, and by ST14 these form the nephric duct and tubules, respectively. We should therefore like to point out that, according to our observations, the nephric duct and tubules form almost simultaneously and that both arise from the cell cord of the intermediate mesoderm. At ST14, the nephric tubules communicate with the nephric duct and open into the body cavity (Fig. 10). Previous workers have noted that the extension of the duct rudiment is caused by the migration and proliferation of cells from its anterior part, rather than by the supply of cells *in situ*

from the adjacent intermediate mesoderm (Martin, 1971), and that duct extension is more rapid than somitogenesis (Jacob et al. 1992). Our morphological observations support these findings, and it is likely that components of the extracellular matrix, such as fibronectin and laminin, influence the migration of the cells of the duct rudiment (Jacob et al. 1991, 1992; Bellairs et al. 1995).

The external glomerulus arises from the epithelium of the nephrostome

The initial appearance of PEGs in the chick embryo has been reported to occur at the level of the 11th to 14th somites at the 30-somite stage (Sedgwick, 1881) or at the level of the 10th to 14th somites at the 32-somite stage (Abdel-Malek, 1950). In our observations of SEM and serial Epon sections, random protrusions of PEGs began to be formed by cells from the epithelium in the medial portion of nephrostomes at the levels of the 8th to 14th somites at ST15 to ST18 (Fig. 10). All PEGs had appeared by about ST18 (Fig. 10) and podocytes and vascular components then differentiate from mesenchymal cells within these PEGs.

The size and number of the EGs in chick embryos have been reported to peak at or after the 36-somite stage (Sedgwick, 1881) or at the 35-somite stage (Abdel-Malek, 1950). Our results, based on measurements of PEGs and EGs in SEM images, revealed no clear differences among PEGs in either size or shape until ST18. Thereafter, differences gradually arose as the stage advanced and PEGs could be divided into two groups after ST21: (1) a small unchanged type that degenerated at the latest by ST28 and (2) a large type, the epithelial cells of which possessed foot processes, that remained after ST28 (Fig. 1). In addition, very large EGs were often partly embedded in the abdominal wall at the level of the 15th to 18th somites in chick embryos from ST26 to ST29 (Fig. 10). Such EGs were said by Sedgwick (1881) and Davies (1950) to result from a union of external and internal glomeruli. We suggest that the union may not take place simply by a fusion of the two types of glomeruli at a later stage but by a fusion of the primordia of glomeruli that arise successively from the epithelium of the nephrostome and from that of the pronephric tubule. One or two large complicated glomeruli were seen (i) at the level of the 14th somite in many embryos until ST22 and (ii) more caudally with advancing stage (Fig. 10), and their relative position may be shifted caudally by the rapid growth of other organs (neural tube, somites, etc.).

The pronephros can be distinguished from the mesonephros after ST17

It has been said that the differentiation of the mesonephros starts on the 3rd day of incubation in the chick embryo (Abdel-Malek, 1950; Stampfli, 1950; Romanoff, 1960) and that the primordia of the glomeruli of the mesonephros appear at ST20 (Narbaitz & Kapal, 1986). However, in human and chick embryos the boundary between the pronephros and mesonephros is unclear (Hoadley, 1926; Keith, 1933; Abdel-Malek, 1950; Hamilton, 1952).

In our observations of longitudinal serial Epon sections, the diameter of the nephric duct at ST17 seemed to differ between the part cranial to the 14th somite and that caudal to the 15th somite, the former being a narrow pronephric duct and the latter a wide mesonephric duct. Moreover, the cranial pronephric tubules were shorter than the mesonephric tubules, and, whereas EGs and IGs frequently coexisted in the pronephros, EGs did not appear in the mesonephros. In addition, from sagittal sections of embryos at successive stages, we confirmed that EGs shifted in the caudal direction with advancing stage and never overlapped with the mesonephros, because EGs and IGs shifted simultaneously. These results suggest that the pronephros and mesonephros can be clearly distinguished.

The pronephros may be functional in chick embryos from ST23 to ST29

It has previously been thought that the pronephros degenerates at an early stage and does not exist in the chick embryo on day 6 of incubation (Abdel-Malek, 1950; Hamilton, 1952). In the present study, however, a few well-developed EGs with vascular tufts were noted at the level of the 13th to 15th somites from ST23 onwards. Their ultrastructure, as reported by Jacob et al. (1977), closely resembled that of the glomerulus of the functional kidney in the young chicken (Pak Poy & Robertson, 1957) and that of the functional mesonephros in the 8-day chick embryo (Gibley & Chang, 1967).

The mesonephros is known to start producing urine after ST25 but before its nephrons have matured fully (Narbaitz & Kapal, 1986). In addition, it has been said that substances produced by the mesonephros increase

the respiratory surface of the embryo by expanding the allantoic sac (Freeman & Vance, 1974). Although the pronephric tubules in chick embryo do not develop well in comparison with those in amphibian larvae (Jaffee, 1963; Christensen, 1964), our observations suggest that, in the chick embryo, the pronephros may function in the same way as the mesonephros for a short period until ST29. Later, the EGs degenerate (although large EGs remain on one or both sides in some embryos from ST30 to ST34) (Fig. 10).

In conclusion, we have revealed in detail the serial development of the pronephros using scanning and transmission electron microscopy, vascular casts and light microscopy of serial Epon sections. The discrepancies in earlier reports (on the canalization of the pronephric tubules and the distinctions between pronephros and mesonephros, etc.) may have been due to confusion as to the correct interpretation of fine details in images of paraffin sections alone. Our use of the SEM probably enabled us to find PEGs at an earlier stage than is possible in paraffin sections. The pronephros, mesonephros and metanephros differ from each other in developmental stage and scale at any given point during ontogeny. However, they seem to be similar in their fundamental developmental processes, as well as being controlled by similar genetic factors and inductive factors, although further study is needed on these points.

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