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A differential screen for genes expressed in the extraembryonic endodermal layer of pre-primitive streak stage chick embryos reveals expression of *Apolipoprotein A1* in hypoblast, endoblast and endoderm

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α-Tropomyosin
Acyl-CoA1 synthetase long-chain 3

ABSTRACT

The lower layer of the pre-gastrulating chick embryo is an extra-embryonic tissue made up of two different cell populations, the hypoblast and the endoblast. The hypoblast is characterized by the expression of inhibitory signalling molecules (e.g. *Cerberus*, *Dickkopf1*, *Crescent*) and others (e.g. *Otx2*, *goosecoid*, *Hex*, *Hesx1/RPX*, *FGF8*). However, no genes expressed in the endoblast have yet been found. We designed a differential screen to identify markers differentially expressed in these two cell populations. This only revealed one novel gene, *Apolipoprotein A1* (*APO A1*) with restricted endodermal layer expression. Expression of *APO A1* begins very early throughout the lower layer (both hypoblast and endoblast). At later stages it is also expressed in the endoderm and its derivatives, the anterior intestinal portal endoderm and the growing liver bud.

1. Results and discussion

Before gastrulation, the chick embryo is a flat disc comprising two layers of cells. The embryo proper will arise from the upper layer, or epiblast, while the lower layer is generally thought only to contribute to extra-embryonic components such as the yok sac stalk (Bellairs, 1953a,b, 1955, 1957). The lower layer is composed of two different cell types: the hypoblast, which forms a layer by the coalescence and expansion of groups of cells initially scattered on the ventral surface of the epiblast, and the endoblast, which grows out from the posterior germ wall margin (a flap of yolky area opaca endoderm, protruding into the marginal zone) (Vakaet, 1962, 1970; Stern, 1990). The endoblast displaces the hypoblast anteriorly, an event that is immediately followed by the emergence of the primitive streak from the posterior epiblast. These tissues appear to have evolved to ensure the formation of a single embryonic axis in an embryo that is otherwise highly regulative: the hypoblast has an inhibitory function, preventing the formation of supernumerary primitive streaks through secretion of Cerberus, a Nodal antagonist. The endoblast, which does not secrete Cerberus, later relieves this inhibition in the posterior part of the embryo and thus contributes to restrict Nodal activity to one end of the blastodisc (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002).

To date there are very few molecular markers that can be used to identify these tissues, especially the endoblast. In an attempt to find new markers, we devised a differential screen between endoblast and hypoblast. Small groups of endoblast and hypoblast cells were isolated from stage 3 (HH; Hamburger and Hamilton, 1951) embryos and a differential screen carried out as previously

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Table 1
The 13 genes identified in the differential screen between hypoblast and endoblast

Gene name (number of clones)	Description
APO A1 (4)	Apolipoprotein A1
ITGB1BP3 (2)	Integrin β-1 binding protein 3
MKRN1	Makorin 1
ERNI	Early response to neural induction
LDHB	Lactate dehydrogenase B
TPM1	α-Tropomyosin
ACSL3	Acyl-CoA1 synthetase long-chain 3
Clone 55	ChEST1023d19 (BX930253)
	Hypothetical LOC430620 (XM_428175)
Clone 99	ChEST928d23 (CR353728)
	ChEST530m4 (CR387775)

The left column gives the abbreviated gene name followed by the number of clones isolated in brackets, when more than one. Clones 55 and 99 do not match any known gene but correspond to the sequences represented by the ESTs/predicted genes shown in the right column.

described (Dulac and Axel, 1995; Streit et al., 2000; Bertocchini and Stern, 2002). One hundred and twenty clones that appeared to be expressed selectively in the endoblast were selected and analysed by in situ hybridisation. Most showed ubiquitous expression and were discarded. The remaining 13 clones were sequenced (Table 1). Four of these encoded the same gene, *Apolipoprotein A1* (*APO A1*). In situ hybridisation of stage XIII-3 embryos confirmed its expression in the endoblast.

We therefore conducted a detailed analysis of the expression of *APO A1* from laying (stage X EG&K; Eyal-Giladi and Kochav, 1976). *APO A1* mRNA first appears in the forming hypoblast of early chick embryo at stage XI EG&K (Fig. 1A and arrow in N). As the hypoblast expands anteriorly, *APO A1* is strongly expressed in the forming lower layer (Fig. 1B and C and arrow in O). From stage XIII, *APO A1* displays asymmetric expression in the lower layer, strongest anteriorly (Fig. 1D and E, and black

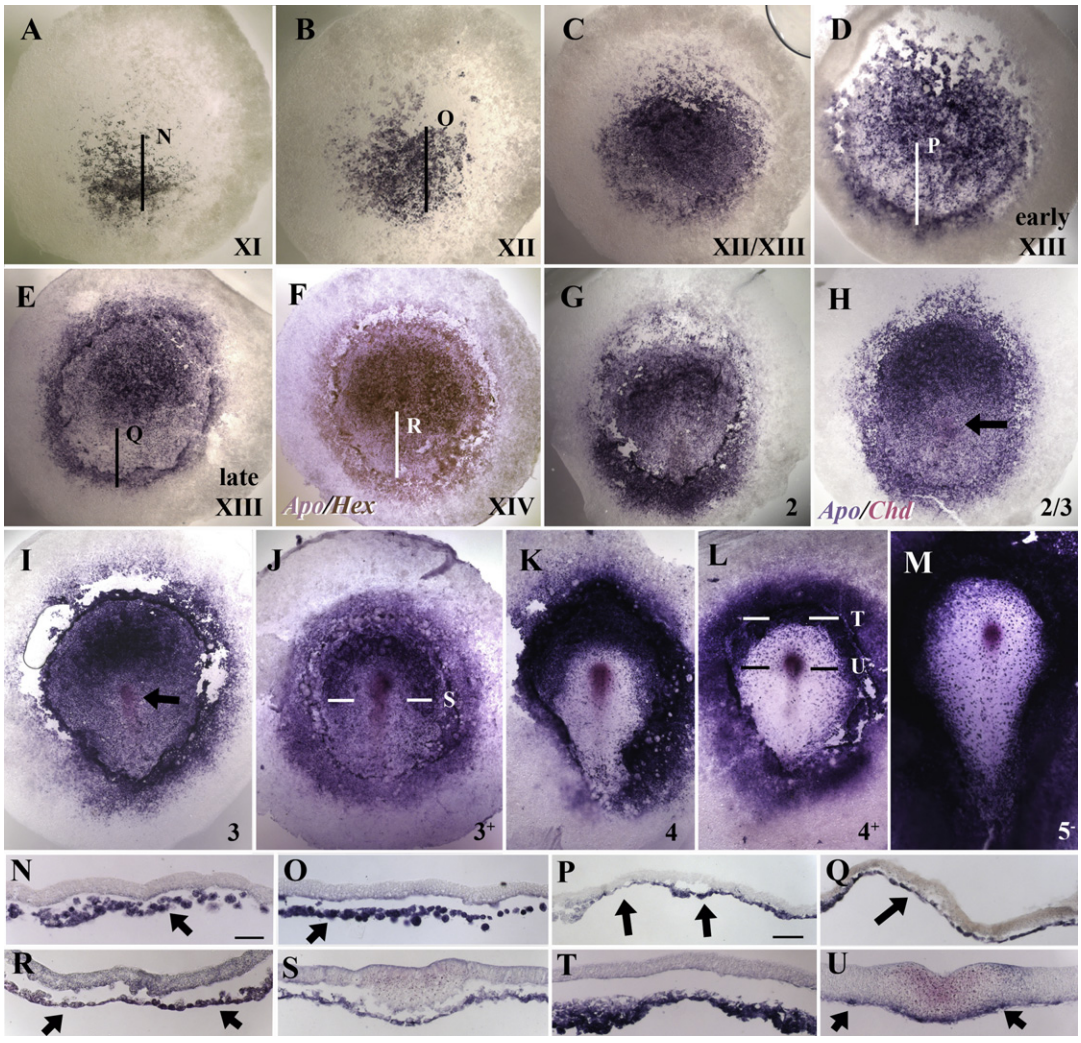

Fig. 1. Expression of *Apolipoprotein A1* (*APO A1*) from stage XI EG&K to stage 5− HH. (A–E and G) Whole mount in situ hybridisation with *Apolipoprotein A1* antisense probe. (F) Double in situ with *APO A1* (purple) and *Hex* (blue). (H–M) Double in situ with *APO A1* (blue) and *Chordin* (purple). All embryos are viewed from ventral side, and are oriented with anterior to the top. Panels (N–U) are paraffin sections (10 μm). (N–R) are sagittal sections oriented with the anterior to the right; (S–U) are transverse sections, as indicated. For panels (N), (O), (Q–U) the scale bar is 50 μm. For panel (P) the scale bar is 100 μm. (A) Stage XI. (B) Stage XII. (C) Stage XII–XIII. (D) Early stage XIII. (E) Late stage XIII. Note in (D and E) the strong *APO A1* expression anteriorly. (F) Stage XIV, after double in situ hybridisation with *APO A1* (red substrate) and Hex (purple substrate). (G) Stage 2. (H) Stage 2/3. Note the early primitive streak growing in the posterior portion of the embryo, with the anterior part expressing *Chordin* (arrow). (I) Stage 3. The anterior primitive streak expressing *Chordin* is indicated (arrow). (J) Stage 3$^+$. (K) Stage 4. The extra-embryonic lower layer is displaced towards the periphery of the embryo by the definitive endoderm. Sparse cells in the embryonic lower layer (definitive endoderm) express *APO A1*. (L) Stage 4$^+$. (M) Stage 5−. Note the cells expressing *APO A1* in the definitive endoderm. (N and O) Sections of the embryos in (A and B), respectively, showing expression in the hypoblast (arrows). (P) Sagittal section (posterior to the left) of the embryo in (D), showing strong expression in anterior regions (right) and weak expression posteriorly (left). The section also shows the morphological difference between the round hypoblast cells (right arrow) and the more posterior flat endoblast cells (left arrow). (Q) Section of the embryo in E showing *APO A1* expression in the endoblast (arrow). (R) Section of the embryo in F showing co-expression of *APO A1* and *Hex* in the hypoblast (right arrow) but only *APO A1* in the endoblast (left arrow). (S) Transverse section of the embryo in J, showing *APO A1* expression in the lower layer. (T) Anterior transverse section of the embryo in L showing strong *APO A1* expression in the extraembryonic lower layer. (U) Transverse section through the embryo in L at the level of Hensen's node, showing *APO A1* expression in sparse cells in the lower layer (arrows).

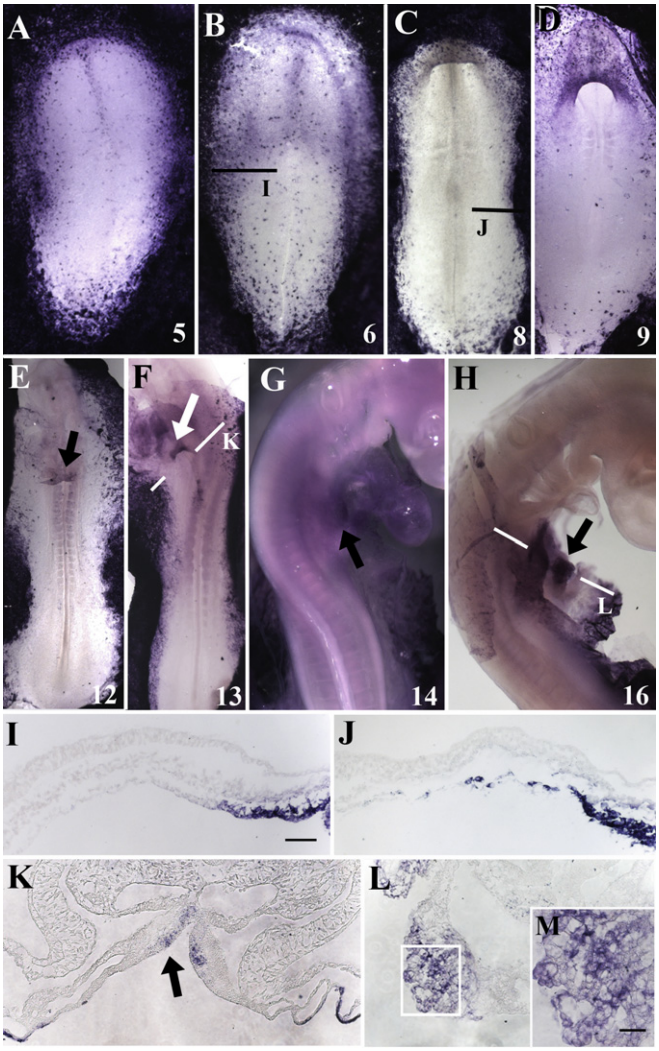

Fig. 2. Expression of *Apolipoprotein A1* (*APO A1*) from stage 5 to stage 16 HH. Panels (A–H) show expression in the whole embryo, panels (I–M) are paraffin sections (10 μm). For panels (I–L) the scale bar I–L is 50 μm. For (M) the scale bar is 20 μm (A and B), stage 5 and 6, respectively, indicating expression in the extra-embryonic lower layer and in isolated cells in the endoderm. (C) Stage 8. (D) Stage 9. (E and F) Stage 12 and 13, respectively, with *APO A1* expression in the Anterior Intestinal Portal (AIP, arrows). (G and H) Stage 14 and 16, respectively, showing expression in the forming liver bud (arrows). (I) Transverse section of the embryo in B, showing expression in the lower layer. (J) Transverse section of the embryo in C. (K) Transverse section of the embryo in F, showing *APO A1* expression in the AIP (arrow). (L) Transverse section of the embryo in H, showing expression in the liver bud. (M) Magnification of the inset in (L).

arrow in P). At around this time, the endoblast starts to invade the lower layer. In the absence of other markers for the endoblast it is difficult to know whether the lower expression seen at early stage XIII corresponds to the earliest endoblast cells or rather to downregulation in the hypoblast. Nevertheless, *APO A1* is expressed in the endoblast, albeit at lower level than in the hypoblast (Fig. 1D–F and P–R). This pattern persists at stage XIV (Fig. 1F). In double in situ hybridisation using the hypoblast marker *Hex* (Yatskievych et al., 1999), *Hex* expression is seen exclusively in the hypoblast (Fig. 1F and right arrow in Fig. 1R), while endoblast cells express only *APO A1* (Fig. 1F and left arrow in Fig. 1R). As the primitive streak appears and elongates (stages 2–4$^+$ HH; Hamburger and Hamilton, 1951), *APO A1* expression is maintained in the lower layer, as it becomes displaced to the germinal crescent anteriorly (Fig. 1G–L and S). In some embryos we performed double in situ hybridisation for *APO A1* and *Chordin* to mark the organizer at the tip of the primitive streak, for reference (Fig. 1H–M, arrows in H and I).

The early lower layer (hypoblast plus endoblast) starts to become displaced by inserting definitive (gut) endoderm cells from stage 4 HH (Bellairs, 1953a,b; Selleck and Stern, 1991; Lawson and Schoenwolf, 2003; Kimura et al., 2006). While still strongly expressed in the hypoblast/endoblast (Figs. 1K–M, T and 2A–D, I and J), *APO A1* is also seen in isolated cells at the centre of the embryo which may correspond to early definitive endoderm cells at stages 4$^+$–6 (Fig. 1K–M, arrows in U, and Fig. 2A and B, arrows in N, O). However, in the absence of completely specific markers for this tissue (Kimura et al., 2006), it is impossible to exclude the possibility that these cells correspond to persisting hypoblast and/or endoblast cells derived from the original lower layer. At stages 6–12, *APO A1* continues to be strongly expressed in the remnants of the early lower layer, and in sparse cells of the definitive endoderm (Fig. 2B–E).

During subsequent stages, *APO A1* starts to be expressed in the Anterior Intestinal Portal (AIP), an anterior fold that expands posteriorly to define the foregut (stage 12–13 HH; Fig. 2E, F and K, arrows in Q, R). While expression is maintained in the hypoblast remnants in the germinal crescent, *APO A1* mRNA is detected also in the forming liver bud from stages 14–16 HH (Fig. 2G, H and L, M). This is consistent with previous reports that *APO A1* is expressed in the liver at E14 (approx. stage 39), followed, just before and after hatching, by expression in other organs such as intestine, kidney, spleen, breast muscle and brain (Byrnes et al., 1987; Ferrari et al., 1987; Rajavashisth et al., 1987).

The expression of Apolipoproteins during vertebrate early development has hardly been studied. There are brief descriptions in zebrafish and mouse (Shi and Heath, 1984; Farese et al., 1996; Babin et al., 1997), but in neither system is there a detailed description of the changes in expression during early development. In zebrafish *Apolipoprotein E* and *A1* are expressed in the yolk syncytial layer at the blastula and gastrula stage, respectively, but no other sites of expression have been described at early stages (Babin et al., 1997). In the mouse, Apolipoprotein A1 protein has been reported in the yolk sac visceral endoderm at 10.5 dpc (Shi and Heath, 1984), while *Apolipoprotein B* transcripts have been described in the same tissue by 9 dpc (Farese et al., 1996). The present study is the first detailed analysis of expression of a member of the Apolipoprotein family during the earliest stages of vertebrate embryonic development. In chick embryos, this is the first gene reported to be expressed in the endoblast, which makes it unlikely that this tissue is made up of dead cells with no function other than displacement of the hypoblast. On the other hand it is puzzling that this exhaustive molecular screen failed to reveal any other genes with specific expression in the endoblast.

Apolipoproteins are proteins bound to a lipid core, specialized in the transport of lipids through the plasma or extracellular fluid (Srivastava and Srivastava, 2000; Willnow et al., 2007). Although their function has been traditionally connected to lipid metabolism homeostasis, it has recently been suggested that they may also act as transporters of lipid-linked morphogens during embryonic development (reviewed in Willnow et al., 2007). This possible role is especially intriguing in the light of several important signalling roles of the hypoblast during early stages of embryonic development, which include: inhibition of multiple axis formation (Bertocchini and Stern, 2002), induction of a "pre-neural plate" state (Albazerchi and Stern, 2007) and regulation of epiblast cell movements during axis formation (Foley et al., 2000; Voiculescu et al., 2007). Gain- and loss-of-function experiments should be designed to investigate these possible functions.

2. Experimental procedures

Fertile hens' eggs were obtained from Henry Stewart & Co. (UK) (Brown Bovan Gold) and Winter egg Farm (Rhode Island Red). Embryos were staged according to Eyal-Giladi and Kochav (1976) (pre-primitive streak stages, in Roman numerals) and Hamburger and Hamilton (1951) (for post-primitive streak stages, in Arabic numerals). A differential screen between small groups of endoblast and hypoblast cells was performed as previously described (Bertocchini and Stern, 2002). *Apolipoprotein A1* was identified by nucleotide BLAST search using NCBI nucleotide database. In situ hybridisation was carried out as previously described (Stern, 1998).

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