Origin of cells giving rise to mesoderm and endoderm in chick embryo

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In amniotes, all of the tissues of the adult arise from the epiblast, one of the two layers of cells present in the early embryo; the mesoderm and gut endoderm arise from an epiblast-derived structure known as the primitive streak. The monoclonal antibody HNK-1[1–3] recognizes the cells of the primitive streak in the chick embryo[4]. Before streak formation, HNK-1 identifies cells that are randomly distributed within the epiblast[4]. We have now used two novel ways to study cell lineage and commitment to show that the epiblast of the early chick embryo contains two distinct populations of cells with different developmental fates at a stage during which 'mesodermal induction' is believed to occur. One cell population, recognized by monoclonal antibody HNK-1, is destined to form mesoderm and endoderm; the rest of the epiblast is unable to give rise to mesoderm if this population of cells is removed.

We designed two simple experiments to investigate whether the HNK-1-positive cells found in the epiblast before primitive streak formation are the precursors of the HNK-1-positive cells of the primitive streak (Fig. 1). In the first experiment, we followed the developmental fate of the HNK-1-positive cells by using a novel approach for cell lineage analysis—the labelling of cells expressing the HNK-1 epitope with the antibody coupled to colloidal gold (Fig. 1*a*). This method enabled us to identify the cells that were HNK-1-positive at the time of labelling regardless of whether or not they continued to express the HNK-1 epitope during subsequent development. After primitive streak formation, virtually all gold-labelled cells were found in mesodermal and definitive-endodermal tissues (Figs 2 and 3). Control embryos labelled with 15-nm colloidal gold, diluted so that random patches of epiblast cells become labelled, showed no differential distribution of labelled cells among the various tissues after embryo culture (Figs 2 and 3). This experiment shows that the HNK-1-positive cells found in the epiblast of the pre-primitive streak (stage XIII; ref. 5) embryo contribute almost exclusively to tissues derived from the primitive streak.

In the second experiment (Fig. 1*b*), we ablated the HNK-1-positive cells of the epiblast by using HNK-1 and complement. When embryos treated in this way were placed in a solution of a dye that is only taken by dead cells, a similar mosaic pattern as that seen by HNK-1 immunocytochemistry was revealed in the epiblast (Fig. 4*d*, *e*). When grown in culture[6], control embryos treated with antibody alone or complement alone developed normally (Fig. 4*a*, *b*), whereas embryos treated with HNK-1 and complement increased in size normally, but had no primitive streak or mesodermal structures (Fig. 4*c*). But treated embryos could be made to develop normally if they received a graft of a piece of primitive streak from an untreated chick or quail embryo—that is, a single primitive streak formed (Fig. 4*f*), and the mesodermal and endodermal cells were derived from the quail graft (Fig. 4*g*). By contrast, treated embryos grafted with a treated quail epiblast or an untreated hypoblast behaved as if they had not been grafted: they failed to form mesodermal structures. This experiment shows that the epiblast cannot form mesodermal structures when the HNK-1-positive cells are removed unless (HNK-1-positive) primitive streak cells are supplied. It further indicates that after removal of these cells, HNK-1-negative cells in the epiblast are not capable of altering their phenotype to become primitive streak cells.

The epiblast of the chick embryo therefore contains a randomly distributed subpopulation of HNK-1-positive cells at a stage (XIII; ref. 5) preceding formation of the primitive streak; these cells give rise to tissues derived from the primitive streak (mesoderm and endoderm).

C. H. Waddington[7] showed that antero-posterior rotation of the hypoblast before primitive streak formation reverses the anterior–posterior axis of the streak. Because the hypoblast coalesces to form a sheet of cells in the posterior–anterior direction, it has been inferred from Waddington's results that

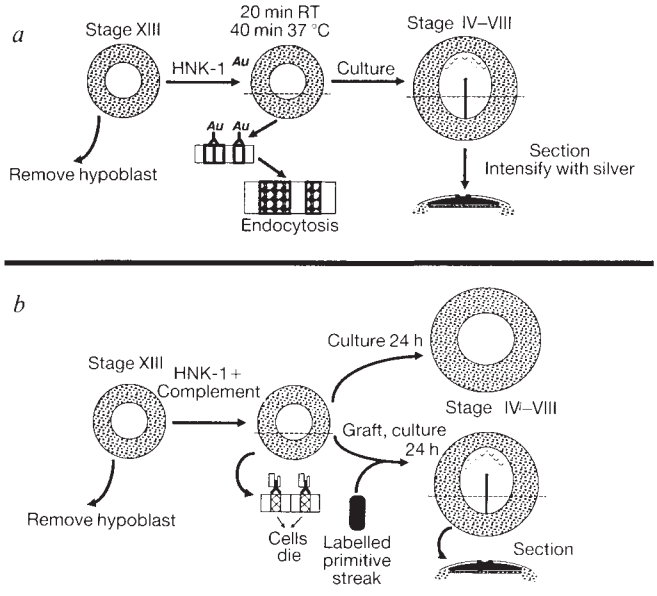

FIG. 1 *a*, Experimental design for lineage mapping of HNK-1-positive cells. The HNK-1-positive cells of the epiblast were marked using the antibody coupled to colloidal gold, and the embryos allowed to develop in culture until after primitive streak formation. Histology then revealed the position of the original HNK-1-positive cells regardless of whether or not they continued to express the epitope. *b*, Ablation of HNK-1-positive cells. In this experiment, the HNK-1-positive cells of the epiblast were killed using the antibody and complement. Some of the treated embryos were grafted with HNK-1-positive cells from a quail donor before further development in culture. Histology then revealed the fate of the transplanted quail cells.
METHODS. *a* Stage XIII (ref. 5) chick embryos deprived of their hypoblast were incubated in HNK-1 covalently coupled to 15 nm colloidal gold (HNK-1^{Au}) for 20 min at room temperature followed by 40 min at 37 °C. During this period the labelled cells endocytose the HNK-1^{Au} complex. They were then washed in Pannett–Compton saline and grown in whole embryo culture to stages 4–8 (ref. 16), formalin-fixed and wax-sectioned and the sections intensified with silver reagent (IntenSE, Janssen). The sections were examined by bright-field microscopy or by confocal laser scanning microscopy in reflection mode. Method for gold-coupling to HNK-1 modified from ref. 17; the optimal pH for adsorption was 8.2. Control embryos treated identically were labelled with 15-nm AuroBeads (Janssen, diluted 1:1 in saline) and then cultured. *b*, Stage XIII (ref. 5) chick embryos deprived of their hypoblast were incubated in HNK-1 supernatant and guinea pig complement (1:1) for 1 h at 37 °C, washed in Pannett–Compton saline, and set up in whole embryo culture[6]. One set of treated and washed embryos received a graft of about one-third of the mesoderm from a stage 3 (ref. 16) primitive streak from a quail embryo after HNK-1–complement treatment and was then cultured. Control experiments: HNK-1 alone (1:1 in saline), complement alone (1:1 in saline). Grafted embryos were fixed in Zenker's fixative and wax-sectioned, and the sections stained with haematoxylin after acid hydrolysis to visualize the quail cells[18,19]. RT, room temperature.

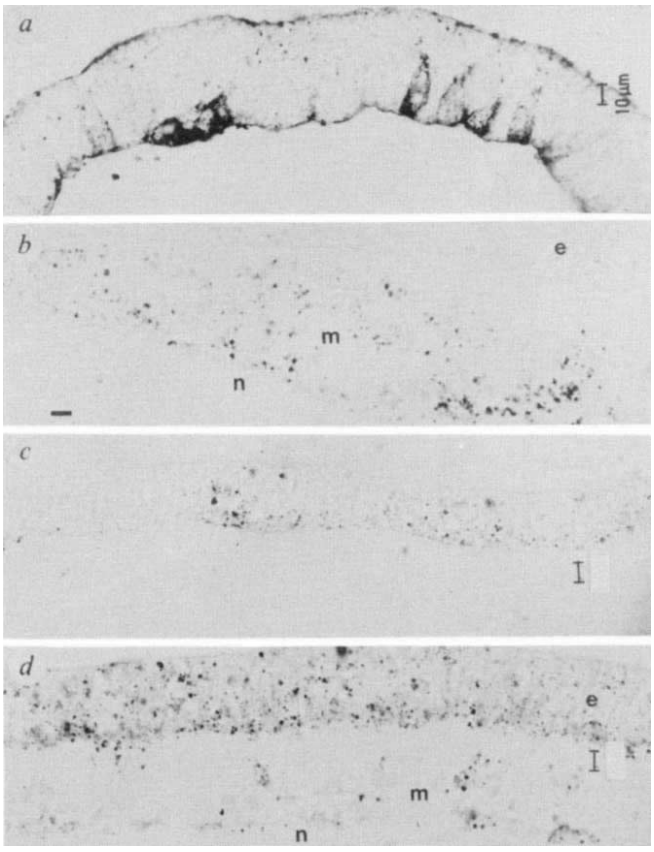

FIG. 2 HNK-1–Au experiment. If embryos were fixed and sectioned immediately after labelling with HNK-1^{Au}, some cells of the epiblast were found to have endocytosed the HNK-1^{Au} complex (*a*). After further incubation, cells labelled with HNK-1^{Au} ($n=3$ (number 31) embryos) were found in the definitive endoderm and mesodermal tissues but not in the ectoderm (*b*); scale bar, 10 μm. Control embryos labelled directly with 15-nm gold particles (*c*; diluted so that only some epiblast cells became labelled) displayed labelled cells in all tissues after a similar period in whole embryo culture (*d*). Abbreviations e, ectoderm; m, mesoderm; n, endoderm.

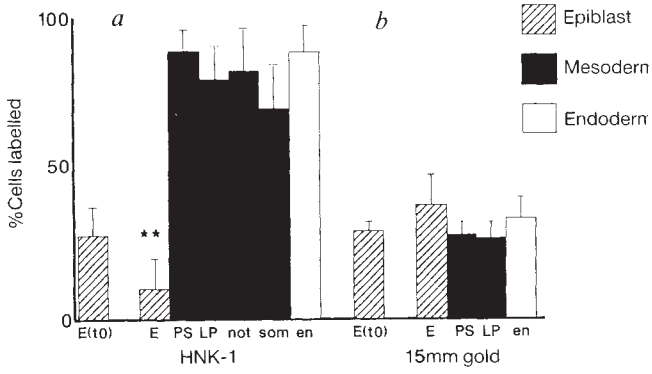

FIG. 3 Quantification of HNK-1–Au experiment. Cells labelled with HNK-1^{Au} were found in mesodermal and endodermal tissues but not in ectoderm. By contrast, cells labelled directly with 15-nm gold were equally distributed among all tissues. The abscissa represents the proportion (%) of labelled cells within a given tissue; counts were obtained from 19 HNK-1^{Au} labelled embryos and five 15-nm-gold-labelled embryos. All of the cells of at least five nonadjacent sections were counted in each embryo. The error bars are standard deviations. E(t0), epiblast immediately after labelling. After culture: E, lateral epiblast; PS, primitive streak (all layers); LP, lateral plate mesoderm; not, notochord; som, somite; en, definitive (gut) endoderm; **, significantly different ($P<0.001$) from each of the other HNK-1^{Au}-labelled tissues and from epiblast labelled with 15-nm gold.

the overlying epiblast is made up of uncommitted cells, and that the hypoblast restricts the fates of the cells with which it first comes into contact at the posterior end of the embryo, so that cells induced by the hypoblast form the primitive streak, whereas uninduced cells remain in the ectoderm and become the precursors of the skin and nervous system[8–12]. The result of Waddington's hypoblast-rotation experiments should be reinterpreted in the light of our findings. Because the HNK-1-positive cells of the epiblast make their appearance in a random distribution among the cells of the epiblast[4], it seems unlikely that the hypoblast induces mesoderm and endoderm from uncommitted epiblast cells with which it first comes into contact at the posterior end of the embryo.

Two populations of cells contribute to the formation of the hypoblast. At first, small islands of 'primary' hypoblast cells are present in the embryo (stage X; ref. 5); these islands are distributed randomly against the ventral surface of the epiblast. Later (stages XI–XIII; ref. 5), these islands receive a contribution from cells ('secondary' hypoblast) derived from the posterior margin of the embryonic disc. These marginal zone-derived secondary hypoblast cells constitute the tissue that has been postulated to induce the primitive streak[9,13–15].

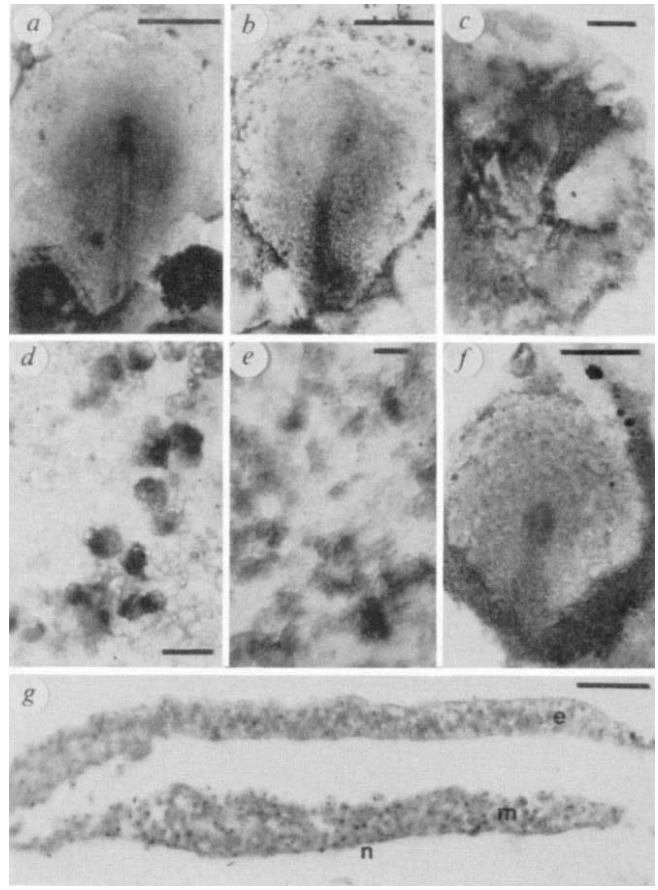

FIG. 4 HNK-1–complement experiment. Embryos treated with HNK-1 antibody alone ($n=10$; *a*) or complement alone ($n=14$; *b*) developed normally, whereas those treated with HNK-1 and complement ($n=32$) failed to form a primitive streak (*c*). HNK-1–complement treatment kills a subpopulation of epiblast cells that can be visualized by brief immersion of the treated embryo in Nigrosin (*d*); the resulting pattern resembles the pattern of staining seen with HNK-1 at this stage (*e*; immunoperoxidase). If a piece of chick ($n=10$) or quail ($n=22$) primitive streak is grafted into embryos treated with HNK-1–complement, a single embryonic axis of normal appearance develops (*f*), and this is composed of donor quail cells (*g*). Scale bars, 500 μm (*a–c, f*); 10 μm (*d, e*); 50 μm (*g*). Abbreviations e, epiblast; m, mesoderm; n, endoderm.

It is possible that in the experiments we performed, the islands of primary hypoblast present during early development induced neighbouring epiblast cells to become primitive streak cells in a very local way at a stage earlier than stage XIII (ref. 5). But it is clear from Waddington's experiment that the secondary hypoblast plays some part in determining the position at which the primitive streak will form, because at stage XIII (ref. 5) the rotation of the secondary hypoblast, or rotation of the marginal zone from which it is derived, reverses the orientation of the embryo[7,13]. So one role of the secondary hypoblast must be to mark the site at which the presumptive primitive streak cells will collect. The hypoblast could also be required for the later differentiation of axial mesoderm cells into notochord and somites. An isolated piece of epiblast from the centre of the embryo cannot form axial structures in the absence of both the marginal zone and the hypoblast, but it can form axial structures if supplied with either of these tissues, even if the hypoblast has been separated into single cells[13–15].

At a stage when the embryo can respond to rotation of the hypoblast or marginal zone by reversal of position of the embryonic axis, the epiblast is composed of a mixture of cells that seem to be committed to become either ectoderm or primitive streak cells. It is therefore clear that the cells of the primitive streak are not induced by the hypoblast at the posterior margin of the embryo at this stage of development. □

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1. Abo, T. & Balch, C. M. *J. Immun.* **127,** 1024 (1981).
2. Tucker, G. C. *et al. Cell Diff.* **14,** 223 (1984).
3. Rickmann, M., Fawcett, J. W. & Keynes, R. *J. J. Embryol. exp. Morph.* **90,** 437 (1985).
4. Canning, D. R. & Stern, C. D. *Development* **104,** 643 (1988).
5. Eyal-Giladi, H. & Kochav, S. *Dev. Biol.* **49,** 321 (1976).
6. New, D. A. T. *J. Embryol. exp. Morph.* **3,** 326 (1955).
7. Waddington, C. H. *Wilheim Roux Arch. EntwMech. Org.* **128,** 502 (1933).
8. Eyal-Giladi, H. *Cell Differ.* **14,** 245 (1984).
9. Azar, Y. & Eyal-Giladi, H. *J. Embryol. exp. Morph.* **61,** 133 (1981).
10. Nieuwkoop, P. D., Johnen, A. G. & Albers, B. *The Epigenetic Nature of Early Chordate Development* (Cambridge University Press, 1985).
11. Smith, J. C. *Development* **105,** 665 (1989).
12. Gurdon, J. B. *Development* **99,** 285 (1987).
13. Azar, Y. & Eyal-Giladi, H. *J. Embryol. exp. Morph.* **52,** 79 (1979).
14. Mitrani, E. & Eyal-Giladi, H. *Nature* **289,** 800 (1981).
15. Mitrani, E. & Eyal-Giladi, H. *Differentiation* **26,** 107 (1984).
16. Hamburger, V. & Hamilton, H. L. *J. Morph.* **88,** 49 (1951).
17. Goodman, S. L. *et al. J. Microsc.* **123,** 201 (1981).
18. Hutson, J. M. & Donahoe, P. K. *Stain Technol.* **59,** 105 (1984).
19. Stern C. D. & Keynes, R. J. *Development* **99,** 261 (1987).

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Identification of classical minor histocompatibility antigen as cell-derived peptide

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HISTOCOMPATIBILITY antigens expressed on tissue grafted between individuals are recognized by host T cells, which reject the graft[1,2]. The major histocompatibility complex (MHC) antigens have been identified on the molecular level, whereas the molecules representing the remaining ones, the minor histocompatibility antigens, are unknown, apart from some exceptions[3–5]. The cytotoxic T lymphocyte (CTL) response against minor histocompatibility antigens shares many aspects with that against virus-infected cells[6,7]. Virus-specific CTL recognize peptides derived from viral proteins produced in the infected cell. These peptides are presented by MHC class I molecules, as indicated by functional and crystallographic data[8,9]. By analogy, minor histocompatibility antigens have been postulated to be peptides derived from normal cellular proteins presented by MHC class I molecules[10–12]. Here we report that peptides derived from normal cellular proteins can indeed be recognized by CTL raised in the classical minor histoincompatible mouse strain combination, C57BL/6 against BALB.B. Thus, we have proven the above postulate, and isolated one of the minor histocompatibility molecules elusive for several decades.

Minor histocompatibility-specific CTL lines (which are widely accepted as a contemporary tool for the detection of minor histocompatibility antigens) were raised by repeatedly stimulating spleen cells from immunized C57BL/6 (abbreviated to B6; H-2^b)-strain mice with multiple minor histoincompatible BALB.B (H-2^b) stimulator cells (the mouse MHC is called H-2, corresponding to the human HLA). Specificity of three independently derived lines and one clone is shown in Table 1. Lines B16X9/1a and B30X9/4a contain MHC class I K^b-restricted CTL and are specific for BALB minor histocompatibility antigens, as shown by recognition of recombinant inbred strains and by F_1 complementation. The third line, B21W9/1a, is specific for an H-4^b determinant expressed on BALB.B cells and is also MHC-restricted. The clone mapki 1, derived from the B16X9/1a line, is K^b-restricted. These CTL were screened for recognition of peptides produced by enzymatic digestion with endoproteinase Lys C (which specifically hydrolyzes peptide bonds at the carboxylic site of lysine) of a protein extract derived from BALB/c spleen cells. CTL were tested on B6-derived EL4 target cells incubated with enzymatic fragments, analogous to the assay system used to define CTL epitopes with synthetic viral peptides[8]. CTL line B16X9/1a, but not B21W9/1a, recognized material eluting at a distinct position of reversed-phase HPLC elution profile (Fig. 1*a*, *b*). Antigenic material from another protein digest was chromatographed on several dimensions including gel filtration (Fig. 1*c*), revealing the relative molecular mass (M_r) of the antigen to be ~1,600 (Fig. 1*d*). In a final separation by reversed-phase HPLC, the antigen eluted as a single sharp peak (Fig. 1*e*, *f*). An attempt to sequence this material indicated that it consisted of more than one peptide species of at most 15 amino acids.

In subsequent experiments, protein extracts derived from BALB.B, B6, or BALB/c spleen, or from the BALB/c-derived B-cell tumour J558, were digested in the same way. The BALB.B, BALB/c and J558 material recognized by B16X9/1a CTL eluted at the same position (around fraction 33) as in Fig. 1*a*, *b*, whereas B6 material at this and adjacent fractions was not recognized (Fig. 2*a*–*d*). CTL line B30X9/4a, but not B21W9/1a, recognized the same active fractions (data not shown). A mock-digest of BALB/c splenic proteins without endoproteinase Lys C, and a self-digest of this enzyme, contained no active fractions (Fig. 2*e*, *f*). Peak fractions 33 of BALB.B, B6, BALB/c and J558 material were retested in titrated amounts. CTL lines B16X9/1a as well as B30X9/4a recognized BALB.B-derived material in a dose-dependent fashion down to a dilution of <1 in 300 (Fig. 3*a*). B6-derived material was not recognized by any of the CTL lines (Fig. 3*b*). Antigenic determinants are of peptidic nature, as proteases of broad specificity, pronase E and proteinase K, destroy these epitopes in BALB/c and J558 material (Fig. 3*c*). The K^b-restricted CTL clone mapki 1 (derived from B16X9/1a, see Table 1) recognizes material from the sharp peptide peak shown in Fig. 1*e* in a dose-dependent manner (Fig. 3*d*). K^b-restricted recognition of minor histocompatibility peptide by

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