

Ontogeny of hyaluronan secretion during early mouse development

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SUMMARY

The ontogeny of hyaluronan (HA) secretion during early mouse embryogenesis has been investigated using a biotin-labelled HA-binding complex from cartilage proteoglycan. HA is first secreted by visceral endoderm cells of the early egg cylinder on day 5.5 post coitum (p.c.), predominantly into the expanding yolk cavity. On day 6.5 p.c., HA is present in both the yolk and proamniotic cavities, but pericellular staining is restricted to the visceral endoderm and a population of embryonic ectoderm cells at the antimesometrial end of the proamniotic cavity. By the primitive streak stage, HA is secreted into the ectoplacental, exocoelomic, amniotic and yolk cavities, whilst the only cells exhibiting pericellular staining are those of the embryonic and extraembryonic mesoderm, including the allantois. Comparisons of HA-staining patterns of cultured whole blastocysts, microdissected trophoderm fragments and immunosurgically isolated inner cell masses, revealed no trophoblast-associated HA secretion during outgrowth *in vitro* but significant synthetic activity by the endodermal derivatives of differentiating inner cell masses. To identify the cell lineages responsible for secretion of HA into the embryonic cavities and to investigate the origin of the HA observed around migrating mesoderm cells, day 7.5 p.c. primitive streak stage conceptuses were dis-

sected into their various embryonic and extraembryonic cell lineages. HA secretion was observed after short-term suspension culture of mesoderm, embryonic ectoderm and embryonic endoderm, but was undetectable in fragments of ectoplacental cone, parietal yolk sac (primary giant trophoblast and parietal endoderm), extraembryonic ectoderm or extraembryonic endoderm. The level of synthesis by the HA-positive tissues was markedly enhanced by culture in medium containing serum, compared with that obtained following culture in medium supplemented with a defined serum substitute containing insulin, transferrin, selenous acid and linoleic acid. This suggests that additional growth factors, present in serum but absent from the serum substitute, are required for optimal HA synthesis by the HA-secreting tissues *in vitro*, and probably also *in vivo*. The implications of these events for implantation and the development of peri- and early post-implantation mouse embryos are discussed, and a new role for HA in the initial formation and expansion of the embryonic cavities is proposed.

Key words: hyaluronan, mouse, embryo, implantation, gastrulation

INTRODUCTION

The extracellular matrix (ECM) glycosaminoglycan (GAG) hyaluronan (HA) and its binding proteins, the hyaladherins, have long been implicated in the regulation of a variety of morphogenetic processes associated with embryogenesis (reviewed, Toole, 1981, 1990; Toole et al., 1984). Due to the structure of the molecule and its hydrophilic nature, HA forms hydrated gels even at very low concentrations (Comper and Laurent, 1978). This property results in expansion of the ECM by the generation of a high osmotic pressure, permitting cell proliferation and migration, and, by reducing contact-mediated cell-cell communication, postponing differentiation (Toole and Trelstad, 1971; Toole et al., 1972; Underhill and Toole, 1981; Kujawa and Tepperman, 1983; Brecht et al., 1986). Removal or reduction of ECM HA in later development is often associated with the onset of differentiation (Toole and Gross, 1971;

Orkin et al., 1985; Kulyk et al., 1987; Toole et al., 1989; Van Straaten et al., 1990).

Since HA lacks an integral protein core and is not known to exhibit any species-specific structural differences, *in situ* detection of the molecule has not been possible using standard immunohistochemical techniques. Early *in situ* studies of HA in embryogenesis at the light microscope level relied on comparative analyses of Alcian blue- or Ruthenium red-stained tissues, following selective digestion of ECM components with specific GAG-degrading enzymes (Kvist and Finnegan, 1970; Bernfield et al., 1972; Derby, 1978; Morriss and Solursh, 1978; Pintar, 1978). However, use of the hyaluronan-binding region of cartilage proteoglycan as a high-affinity HA-specific probe has greatly facilitated the detection of HA *in vivo* and *in vitro* (Ripellino et al., 1985; Green et al., 1988). Whilst investigating the role of HA in mouse uterine differentiation and trophoblast invasion during implantation, using the biotinylated proteoglycan

probe described by Green et al. (1988), we observed that certain tissues of the implanting conceptus began secreting HA as early as day 5.5 p.c. (Brown and Papaioannou, 1992). Since the pattern of HA expression prior to differentiation of the three primary germ layers (ectoderm, endoderm and mesoderm) has not previously been examined histochemically in mammals, and since former *in situ* studies began after primitive streak formation on day 8.5 p.c. in the mouse (Van Straaten et al., 1990) and day 9.5 p.c. in the rat (Morriss and Solursh, 1978; Poelman et al., 1990), it was decided to examine the ontogeny of HA secretion in the early mouse embryo from implantation (day 4.5 p.c.) to the onset of organogenesis (day 8.5 p.c.).

MATERIALS AND METHODS

Tissue collection

Oestrus-selected CD-1 female mice (Charles River, Cambridge, MA) were mated overnight with CD-1 males and pregnancy confirmed by vaginal plug detection the following morning. Uteri were dissected from pregnant females at daily intervals between 4.5 and 8.5 days p.c., assuming midnight to be the time of coitus. To optimise structural preservation of the tissues, embryos were fixed in the intact uterus (days 4.5 and 5.5 p.c.) or in dissected decidual swellings (days 6.5 to 8.5 p.c.). Explanted tissues were washed briefly in modified PB-1 medium (mPB-1; Papaioannou and West, 1981) containing 10% foetal bovine serum (FBS; Hyclone, Logan, UT) and fixed overnight at 4°C in 4% paraformaldehyde/0.1 M phosphate buffer containing 0.5% cetylpyridium chloride (CPC). Although fixative containing CPC induced the formation of membranous vesicles in many of the intercellular spaces, as found previously (Morriss and Solursh, 1978), omission of this compound resulted in a complete loss of HA from the embryonic cavities during histological processing (data not presented). Fixative containing CPC was therefore used throughout these studies, despite its somewhat disruptive influence on the morphologic preservation of certain tissues. Fixed tissues were washed for 24 hours at room temperature in 0.25 M sucrose/0.2 M glycine in 0.1 M phosphate buffer. After dehydration in alcohol and clearing in xylene, tissues were Paraplast (Oxford Labware, St Louis, MO) embedded and 5 µm serial sections cut and mounted on gelatin-coated slides. Four to twelve different embryos from a minimum of two mice were examined at each developmental stage: 4 embryos on day 4.5 p.c., 5 on day 5.5 p.c., 12 on day 6.5 p.c., 5 on day 7.5 p.c., and 4 on day 8.5 p.c.

Blastocyst collection, microdissection, immunosurgery and culture

Early expanded blastocysts were flushed from the uteri of pregnant mice (10.00-12.00 hours, day 3.5 p.c.) using mPB-1 medium + 10% FBS, and placed singly in hanging drops of the same medium in a Leitz micromanipulator chamber. Mural trophoblast cells were dissected away from the inner cell mass (ICM) and its overlying polar trophoblast, using two solid glass needles as described by Papaioannou (1981), except that the micromanipulation chamber was maintained at 6°C on a cooled microscope stage (Sensortek, Clifton, NJ) to reduce the stickiness of the dissected cells. Isolated ICMs were obtained by immunosurgical removal of trophoblast cells from whole blastocysts (Solter and Knowles, 1975). Briefly, blastocysts, which had had their zonae removed by brief exposure to acid-Tyrodes (Hogan et al., 1986), were incubated in rabbit anti-mouse serum, diluted 1:10 in mPB-1, for 30 minutes at 37°C. After washing thoroughly in

mPB-1 + 10% FBS, embryos were incubated for 30 minutes in guinea pig complement (Pel-Freez Biologicals, Rogers, AR), diluted 1:10 in mPB-1. Lysed trophoblast cells were gently pipetted away from the intact ICM using a finely drawn Pasteur pipette. Isolated trophoblast fragments, ICMs, and zona-free and zona-intact whole blastocysts were cultured in separate wells of glass tissue-culture chamber slides (Nunc, Naperville, IL) in DMEM (Sigma) + 10% FBS at 37°C, in a humidified atmosphere of 10% CO₂ in air. Outgrowths were fixed after culture for 2 to 4 days and processed for histochemical localization of HA as described below.

Dissection and culture of day 7.5 p.c. embryos

In eight separate experiments, decidual swellings containing embryos were obtained from the uteri of pregnant females on day 7.5 p.c. and dissected essentially as described by Beddington (1987) and Tam (1990). After peeling open the decidua with two pairs of fine watchmakers forceps, intact egg cylinders were shelled out into mPB-1 + 10% FBS. To standardize the developmental stage of the cultured tissues, only Theiler stage 10 embryos were selected for dissection, and any earlier or later stages discarded (Theiler, 1989; Kaufman, 1992). The parietal yolk sac (PYS) was first peeled away with fine forceps and set aside for culture. The ectoplacental cone (EPC) was dissected from the abembryonic pole of the egg cylinder with a pair of finely drawn solid glass needles, and the diploid core isolated by scraping away the surrounding secondary giant trophoblast and contaminating maternal blood. After cutting the remaining egg cylinder in two at the level of the amniotic fold, taking care to discard material from the embryonic/extraembryonic junction, embryonic and extraembryonic tissues were isolated by enzymatic separation of the tissue layers using a 2.5% pancreatin/0.5% trypsin solution (Levak-Svajger et al., 1969). Embryonic and extraembryonic tissues were incubated for 1 hour and 10 minutes, respectively, in the enzyme solution at 4°C, and washed several times in mPB-1 + 10% FBS. Tissue layers were separated by repeatedly pipetting the fragments in a finely drawn Pasteur pipette with bore diameter slightly smaller than the diameter of the fragments. Extraembryonic ectoderm (ExEc), endoderm (ExEn) and mesoderm generally separated cleanly following this treatment; however, embryonic tissues remained attached in the region of the primitive streak and had to be teased apart using fine glass needles. Due to the risk of contaminating embryonic ectoderm (EmEc) and endoderm (EmEn) with mesoderm from the streak, only clearly separated layers obtained from the opposite side of the egg cylinder were selected for culture (Beddington, 1987). Isolated tissues were either fixed immediately, or cultured for 24 or 48 hours in suspension culture in DMEM + 10% FBS on bacteriological grade plastic dishes (VWR, Boston, MA) at 37°C in a humidified atmosphere of 10% CO₂ in air. This system was chosen to prevent attachment of the fragments to the surface of the dish, thereby minimizing the degree of cell differentiation during culture (Dziadek and Adamson, 1978; Tam, 1990). Isolated tissue fragments were also cultured for 48 hours in DMEM + ITSTM serum substitute (6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenous acid, 5.35 µg/ml linoleic acid, 1.25 mg/ml bovine serum albumin; Collaborative Research, Bedford, MA) to control for the possibility that serum-derived HA adsorption by the tissues might lead to misinterpretation of the data. As a final control, isolated mesoderm, EmEc and EmEn fragments, obtained from 20 embryos, were cultured for 48 hours in DMEM + ITSTM to which bovine tracheal HA (Sigma) had been added at a final concentration of 0.01 mg/l. Following culture, the fragments were fixed and embedded in 1.5% agarose/0.1 M phosphate buffer, using a modification of the technique of Yuan and Gulyas (1981), which replaces cacodylate with phosphate buffer, to facilitate handling during paraffin embedding.

Histochemical localization of HA

Detection of HA was achieved using a biotinylated fragment of bovine nasal cartilage proteoglycan (b-PG) which binds with high affinity and specificity to HA (Ripellino et al., 1985). Preparation of the b-PG probe, a gift of Dr. Charles Underhill, has been described previously (Green et al., 1988). Staining was performed in accordance with previously published protocols (Green et al., 1988; Brown and Papaioannou, 1992). Briefly, sections and/or whole mounts of cultured cells were incubated at room temperature for 1 hour with the b-PG probe (2 µg/ml) in 90% calcium/magnesium-free PBS + 10% FBS, and the label detected using a streptavidin/horse radish peroxidase complex (Kirkegaard and Perry, Gaithersburg, MD). Peroxidase activity was visualized by incubation in a solution of 0.02% 3-amino-9-ethylcarbazole (AEC; Sigma)/0.03% H₂O₂ in 0.05 M sodium acetate buffer. Slides were counterstained in Mayer's haematoxylin (Sigma) and mounted in Crystal Mount (Biomedica, Foster City, CA) to preserve the red AEC staining. After drying, permanent slides were prepared with Permount (Fisher Scientific, Medford, MA) and the specimens photographed on an Olympus photomicroscope using Kodak Ektar 25 colour print film.

In each experiment, one of three different controls was always included: consecutive slides of serially sectioned tissue, and/or duplicate whole mounts of cultured tissues, were either (1) predigested with bovine testicular hyaluronidase (100 i.u./ml, 20 minutes, room temperature) before addition of the b-PG probe, or (2) coincubated with probe in the presence of bovine testicular hyaluronidase (100 i.u./ml) or (3) *Streptomyces* hyaluronidase (40 i.u./ml), which exclusively degrades HA (Ohya and Kaneko, 1970; Derby and Pintar, 1978).

RESULTS

HA distribution in the early mouse embryo in vivo

No embryonic HA was detected on day 4.5 p.c. in the attaching blastocyst (Fig. 1A). HA staining was first observed in the yolk cavity of the early egg cylinder on day 5.5 p.c. Visceral endoderm cells also appeared HA-positive, but precise tissue localization was impossible due to the small size of the tissues at this stage, the extracellular location of the molecule and the formation of CPC-induced vesicles in the intercellular space (Fig. 1B). On day 6.5 p.c., strong staining was observed in both the yolk and proamniotic cavities. Pericellular staining was observed in the visceral extraembryonic endoderm and apical pericellular staining was observed in association with embryonic ectoderm cells at the antimesometrial pole of the proamniotic cavity (Fig. 1C,D). By the primitive streak stage (day 7.5 p.c.), HA was detected in the ectoplacental, exocoelomic, amniotic and yolk cavities. The only cells exhibiting distinct pericellular staining were those of the embryonic and extraembryonic mesoderm, including the allantois (Fig. 1E). On day 8.5 p.c. strong positive reactivity was observed within all the embryonic cavities and all mesodermally derived mesenchyme, with the sole exception of the somites, whereas neurectoderm cells were negative (Fig. 1F). No nonspecific staining was detected in any of the hyaluronidase-treated control sections (data not shown, however see figure 1A, Brown and Papaioannou, 1992).

Table 1. HA expression by whole blastocysts, dissected trophoderm fragments and isolated ICMs in vitro

Tissue	Duration of culture (h)	No. of embryos	No. of outgrowths	
			HA-positive	HA-negative
Whole blastocysts	48	18	18	0
	72	23	23	0
	96	33	33	0
		74	74	0
Trophectoderm	48	6	2*	4
	72	11	0	11
	96	5	2*	3
		22	4*	18
ICM	48	9	9	0
	72	6	6	0
	96	5	5	0
		20	20	0

*Contaminating ICM derivatives observed on outgrowths.

HA secretion by whole-blastocyst outgrowths, microdissected trophoderm fragments and immunosurgically isolated ICMs in vitro

Whole-blastocyst outgrowths stained positively for HA after 48 hours in culture (Table 1; Fig. 2A). No differences in the pattern of staining were observed after culture for 48, 72 or 96 hours, although the extent of the outgrowth increased with time. Similarly, no differences were observed in the pattern of staining exhibited by zona-free or zona-intact whole-blastocyst cultures, so the data from both were pooled. Strongest staining was observed in the immediate vicinity of the ICM and its associated endoderm derivatives, although some HA-positive material was also detected on the outgrowing trophoblast (Fig. 2A). To determine whether trophoblast-associated HA was derived from trophoblast secretion or secretion by cells from other lineages, trophoderm fragments dissected from early expanded blastocysts were cultured and stained in a similar manner. No HA secretion was detected in any of the fragments containing trophoblast cells only (Fig. 2C), however 4 of the 22 outgrowths did stain positively for HA (Table 1). All four of these outgrowths contained small ICM-like cell aggregates on their dorsal surface, so it was concluded that these cultures included contaminating ICM derivatives, resulting from incomplete blastocyst microdissection. All of the cultured ICMs stained strongly for HA after culture (Fig. 2B; Table 1), confirming the origin of the HA secretion observed in whole-blastocyst outgrowths.

HA detection in dissected tissue fragments from day 7.5 p.c. embryos in vitro

Several different patterns of HA expression were observed in the dissected tissue fragments of day 7.5 p.c. embryos (Table 2; Fig. 3). Ectoplacental cone (EPC) cells consistently failed to stain for HA, either at the time of dissection or after culture in serum-containing medium for 24 or 48 hours. At the time of dissection, small pockets of HA were detected, trapped within the cavities of dissected parietal yolk sac (PYS) fragments, but no staining was observed in association with either the large giant trophoblast or

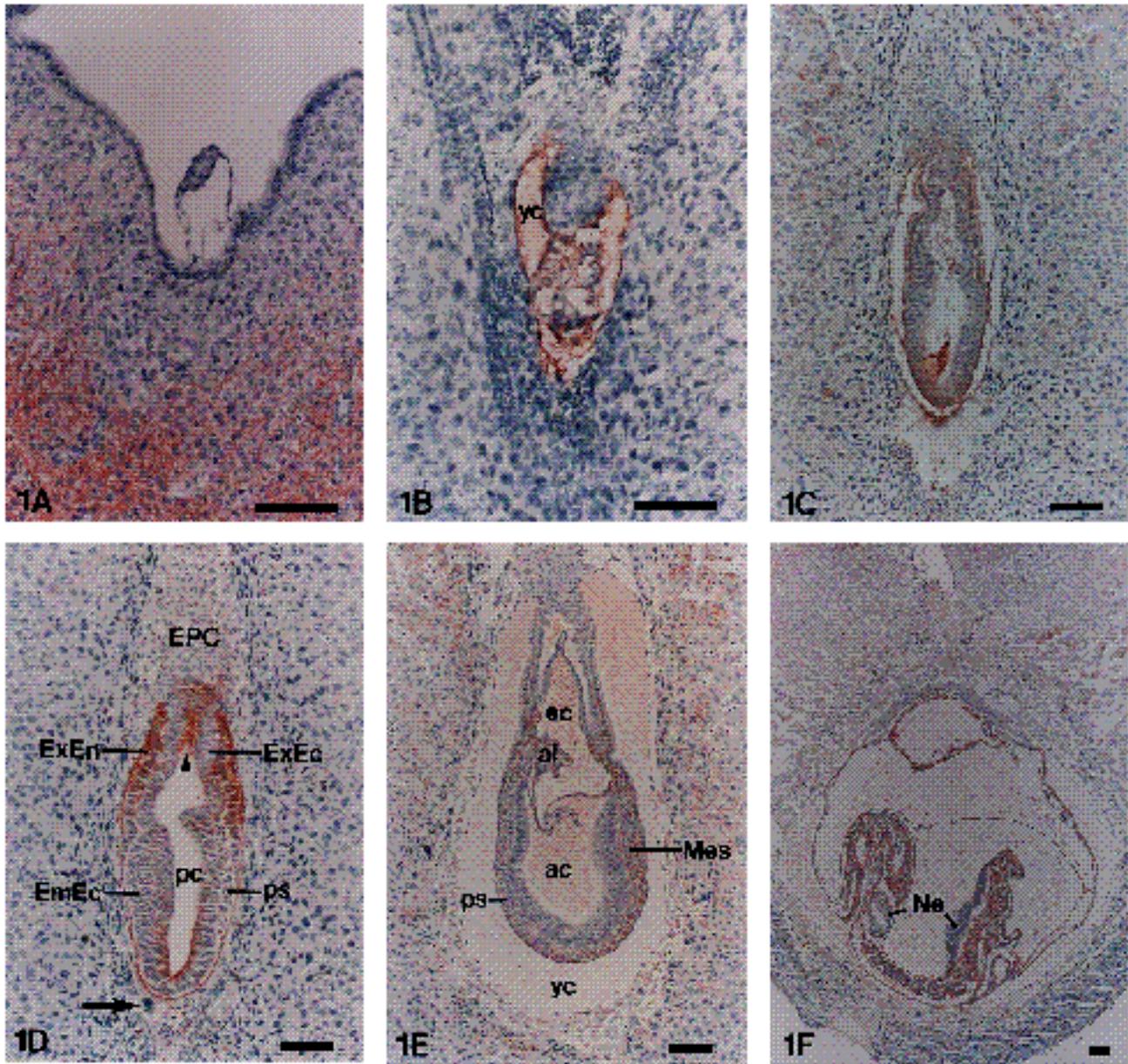


Fig. 1. Expression of HA (red) by peri- and early post-implantation mouse embryos in vivo. (A) In contrast to the underlying nondecidualized endometrium (bottom), no HA-positive staining is detected in an attachment-phase blastocyst (centre) on day 4.5 p.c. (B) Intense positive staining within the yolk cavity of an implanting egg cylinder on day 5.5 p.c. (C) Egg cylinder stage (day 6.5 p.c.). Light pericellular staining observed around visceral endoderm cells and embryonic ectoderm cells at the antimesometrial end of the proamniotic cavity. Positive reactivity exhibited by secreted material within the proamniotic cavity. (D) Early primitive streak stage (day 6.5 p.c.). Strong staining around visceral endoderm cells, and the apical aspect of embryonic ectoderm cells at the antimesometrial end of the proamniotic cavity. Cells entering the streak are clearly HA negative at this stage. Retention of secreted mucopolysaccharide in the proamniotic cavity has been achieved in the mesometrial region only (arrowhead). No staining was ever observed in association with invading giant trophoblast cells in vivo (example arrowed in D). (E) Positive reactivity detected within all embryonic cavities at the primitive streak stage on day 7.5 p.c. Pericellular staining restricted to migrating mesoderm cells, including those of the allantois. (F) On day 8.5 p.c., strong positive reactivity is detected within the mesodermally derived mesenchyme, whilst neurectoderm cells are negative. Reduced staining is associated with differentiation of the somites. Longitudinal (A,B) and transverse (C-F) uterine sections. Mesometrial, top; antimesometrial, bottom. EPC, ectoplacental cone; ExEc, extraembryonic ectoderm; ExEn, extraembryonic endoderm; EmEc, embryonic ectoderm; Mes, mesoderm; Ne, neurectoderm; al, allantois; yc, yolk cavity; pc, proamniotic cavity; ac, amniotic cavity; ec, exocoelomic cavity; ps, primitive streak. Bar, 100 μ m.

small parietal endoderm cells following culture. Extraembryonic ectoderm (ExEc), like its progenitor EPC, failed to stain for the presence of HA at any stage, with the excep-

tion of 5 of the 25 fragments cultured for 48 hours. Since the positive staining observed in these 5 fragments was always associated with a minority subpopulation of cells,

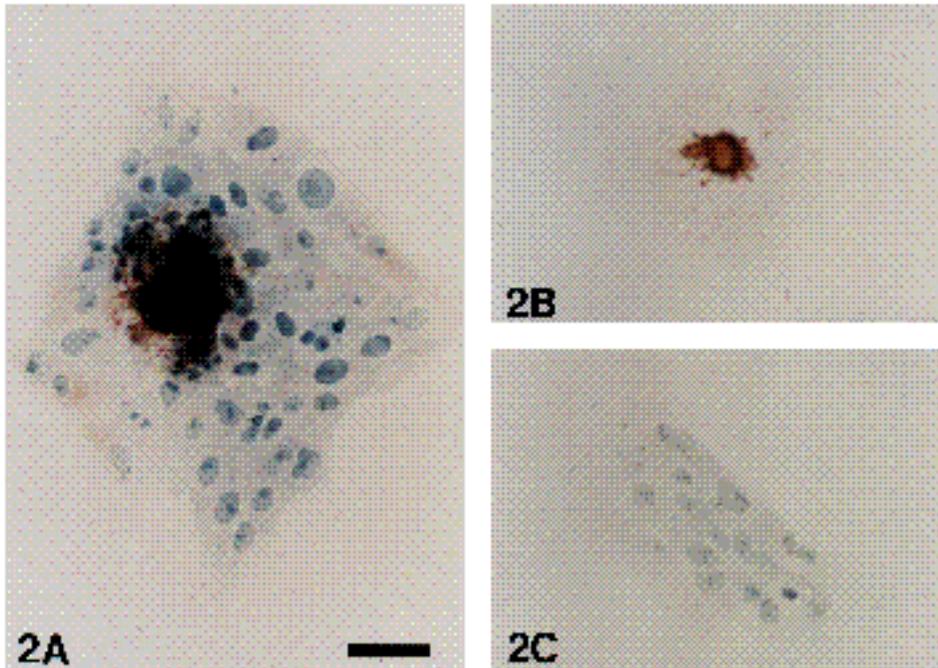


Fig. 2. (A) Detection of HA (red) on an outgrowing whole blastocyst cultured for 96 hours; strongest staining observed around the centrally located ICM. (B) Immunosurgically isolated ICM cultured for the same period exhibits intense positive reactivity, whereas (C) microdissected trophoblast cells lack any histochemically detectable HA. Bar, 100 μ m.

Table 2. HA expression by cultured tissue fragments following dissection of day 7.5 p.c. mouse embryos

Tissue	DMEM + 10% FBS						DMEM + ITS+ TM serum substitute	
	0 hours ^a		24 hours ^a		48 hours ^b		48 hours ^a	
	HA+	HA-	HA+	HA-	HA+	HA-	HA+	HA-
EPC	0	14	0	16	0	25	0	19
PYS	18	0	0	12	0	22	0	21
ExEc	0	13	0	24	5*	20	0	18
ExEn	18	0	2*	20	1*	27	1**	23
Mes	14	0	15	1**	25	1**	23 ⁺	0
EmEc	1	16	4	0	20	0	17 ⁺	0
EmEn	3	10	10	0	27	0	19 ⁺	0

Results show the number of fragments that were HA positive (HA+) or HA negative (HA-) after various culture periods in the two different media.

^aData generated from a single experiment.

^bData pooled from four replicate experiments.

*Contaminating mesoderm present.

**Misclassified ExEc.

⁺Staining intensity markedly reduced compared with that observed in similar fragments cultured in the presence of serum.

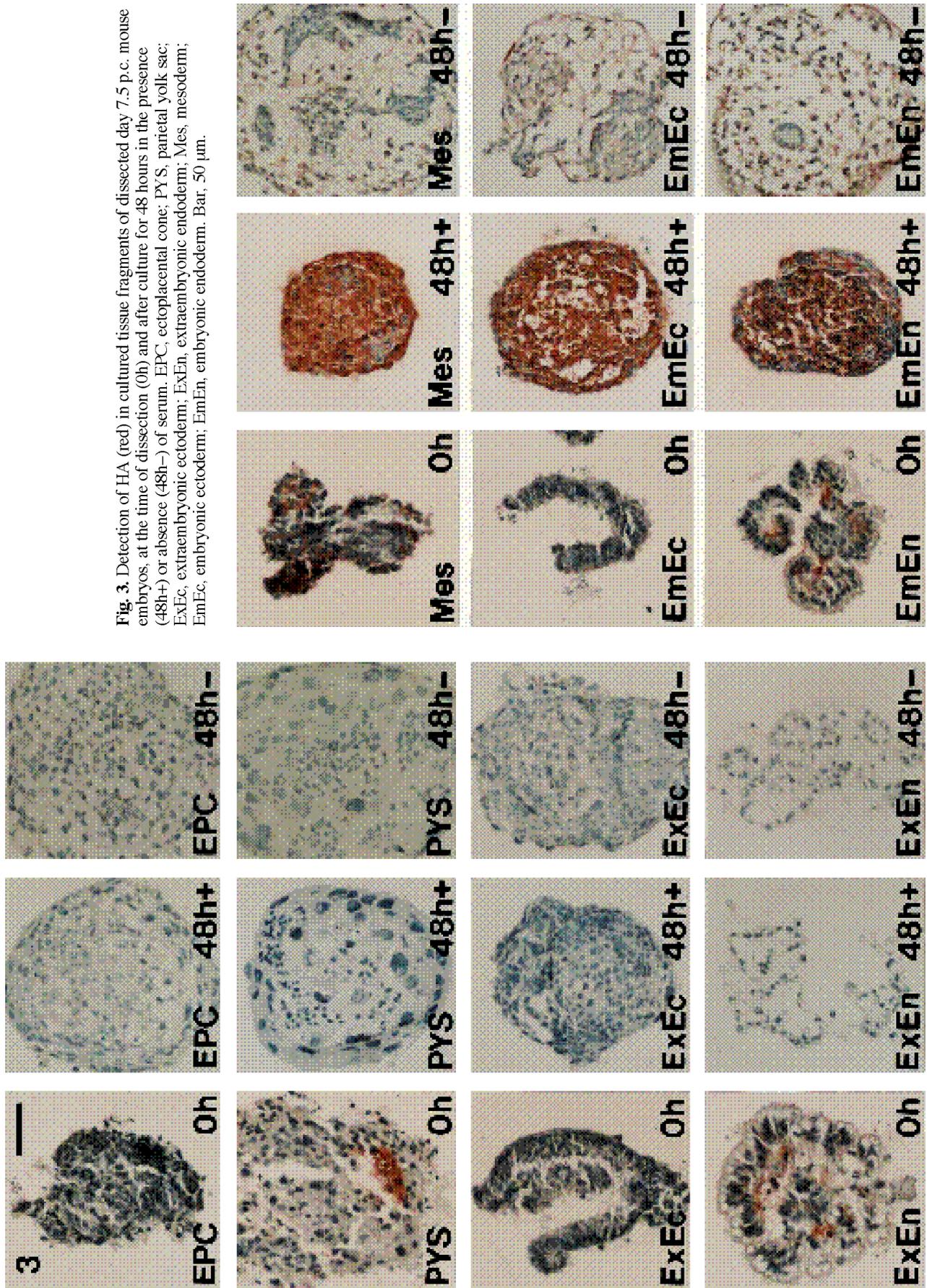
EPC, ectoplacental cone; PYS, parietal yolk sac; ExEc, extraembryonic ectoderm; ExEn, extraembryonic endoderm; Mes, mesoderm; EmEc, embryonic ectoderm; EmEn, embryonic endoderm.

this appeared to represent contamination by mesoderm, resulting from incomplete tissue separation following enzyme digestion. Visceral extraembryonic endoderm (ExEn), like the PYS, stained faintly for HA at the time of dissection, but mostly failed to stain following culture. The only exceptions to this pattern were 3 of the 50 cultured fragments, which stained positively for HA and were similarly deemed to contain contaminating mesoderm cells. Mesoderm fragments, which initially stained weakly for HA, stained intensely after culture for 24 or 48 hours. Only 2 of the 56 presumptive mesoderm fragments examined failed to stain. Since dissected ExEn is easily distinguished visually from mesoderm, it was determined that both these fragments probably consisted of ExEc, misclassified as mesoderm at the time of dissection. The pattern of HA staining observed in both EmEc and EmEn was essentially

the same. Immediately after dissection little or no staining was detected in either tissue; however, increasingly intense staining was observed after culture for 24 or 48 hours.

Since serum itself contains low (0.01-0.1 mg/l) but detectable levels of HA (Laurent and Fraser, 1986), increases in staining intensity during culture might result from HA adsorption, rather than secretion, by the tissues. Dissected tissue fragments were therefore cultured in DMEM + ITS+TM serum substitute for 48 hours before fixation and staining for HA (Table 2; Fig. 3). Qualitatively, the pattern of expression exhibited by each of the cultured tissues was identical to that observed after culture in medium containing serum. EPC, PYS, ExEc and ExEn all failed to stain for HA following culture, whereas mesoderm, EmEc and EmEn stained positively in both the presence or absence of exogenous HA. It should be noted, however,

Fig. 3. Detection of HA (red) in cultured tissue fragments of dissected day 7.5 p.c. mouse embryos, at the time of dissection (0h) and after culture for 48 hours in the presence (48h+) or absence (48h-) of serum. EPC, ectoplacental cone; PYS, parietal yolk sac; ExEc, extraembryonic ectoderm; ExEn, extraembryonic endoderm; Mes, mesoderm; EmEc, embryonic ectoderm; EmEn, embryonic endoderm. Bar, 50 μ m.



that the staining intensity of the HA-positive tissues was markedly lower than that observed in similar fragments cultured for the same period in the presence of serum (Fig. 3). Since the staining intensity of the HA-positive tissues in these serum-free cultures was the same in both the presence or absence of exogenous HA, HA adsorption was not responsible for the enhanced staining exhibited by tissues cultured in the presence of serum.

DISCUSSION

HA expression and trophoblast invasion

We have recently shown that periimplantation differentiation of the mouse endometrial stroma (decidualization) is accompanied by clearance of HA from the ECM, and conclude that this phenomenon serves to restrict trophoblast invasion, by reducing the hydration of the matrix and allowing enhanced adhesive interactions between adjacent decidual cells (Brown and Papaioannou, 1992). However, Carson et al. (1987) implicated HA in promoting trophoblast invasion by showing that HA-coated tissue-culture plastic supports attachment and spreading of preimplantation blastocysts cultured in serum-free medium, whereas uncoated plastic does not. Two hypotheses have been advanced to resolve these apparently contradictory observations (Brown and Papaioannou, 1992). Firstly, cell surface receptors for HA typically form multipoint attachments, recognizing a variety of different matrix components and cell surface ligands (Turley and Moore, 1984; Aruffo et al., 1990; Gupta et al., 1991). Consequently, matrix receptors expressed by invading trophoblast for its interaction with genuine extracellular epitopes *in vivo*, might also be able to interact with inappropriate (nonphysiological) substrata, including HA, *in vitro*. Secondly, trophoblast forms a non-apical but nevertheless polarized epithelium *in vivo*, whilst culture on plastic results in the complete loss of polarity (Enders et al., 1981). Matrix receptors involved with adhesion to the trophoblastic basement membrane (Reichert's membrane) *in vivo*, would therefore come to be expressed indiscriminately over the entire cell surface during outgrowth *in vitro*, leading to possible misinterpretation of the data. Another possibility is that trophoblast itself, like many metastatic cells (Toole et al., 1979; Iozzo, 1985; Turley, 1992), synthesizes HA to facilitate invasion of the HA-negative decidualized stroma. Several lines of evidence presented here now rule out all but the first of these hypotheses. No HA-positive material was detected in the basement membrane separating mural trophoblast from the underlying parietal endoderm in the periimplantation blastocyst (Fig. 1A), or around invading giant trophoblast cells *in vivo* (example arrowed in Fig. 1D). Additionally, no trophoblast-derived HA was detected in isolated trophoblast (Fig. 2C) or PYS fragments (Fig. 3) cultured *in vitro*. Together, these observations confirm that trophoblast cells are not exposed to extracellular HA *in vivo*, either basolaterally, with respect to their underlying basement membrane, or contralaterally, with respect to their interface with the adjacent decidualized endometrium. The ability of trophoblast to attach to and spread on HA-coated plastic *in vitro* (Carson et al., 1987) therefore appears to result from the

presence of matrix receptors which are functionally expressed for interactions with other ECM epitopes *in vivo*; potential candidates include fibronectin, laminin and collagen (Turley and Moore, 1984; Gupta et al., 1991; Faassen et al., 1992; Jalkanen and Jalkanen, 1992), all known to be present in the ECM of decidualized stroma (Wewer et al., 1986; Glasser et al., 1987; Karkavelas et al., 1988; Senior et al., 1988).

Origin and function of HA in the embryonic cavities

HA is first secreted into the expanding yolk cavity on day 5.5 p.c. (Fig. 1B). This cavity is bounded by two distinct cell types: parietal endoderm, lining the blastocoelic surface of Reichert's membrane; and visceral endoderm, enveloping the epiblast. No HA synthetic activity was ever detected in parietal endoderm cells, either during migration from the primitive endoderm in the blastocyst (Fig. 1A), or following culture of dissected PYS fragments (Fig. 3). However, substantial amounts of HA were detected following *in vitro* differentiation of isolated ICMs (Fig. 2B), during which endodermal cells exhibiting a predominantly visceral phenotype are generated (Pedersen et al., 1977; Hogan and Tilly, 1978). Together, this evidence suggests that it is visceral, rather than parietal, endoderm that is responsible for secretion of HA into the yolk cavity on day 5.5 p.c. This idea is further supported by the detection of significant pericellular HA around proliferating visceral extraembryonic endoderm cells one day later (Fig. 1C, D).

HA in the proamniotic cavity on day 6.5 p.c. appears to derive from the synthetic activity of EmEc cells, predominantly those at its antimesometrial end (Fig. 1D). Supporting evidence stems from the fact that little or no pericellular HA was detected in ExEc *in vivo* (Fig. 1C, D), and none following culture (Fig. 3), whereas EmEc stained positively for HA both *in vivo* and *in vitro*. The possibility that ExEc contributes to the secretion of proamniotic HA *in vivo* cannot be excluded however, as suspension culture of this isolated tissue results in its reversion to a diploid trophoblastic phenotype, followed by giant cell transformation (Rossant and Ofer, 1977; Ilgren, 1981; Johnson and Rossant, 1981; Rossant and Tamura-Lis, 1981), and no trophoblastic cell type synthesized HA *in vivo* (Fig. 1) or *in vitro* (Figs 2C, 3) in the present study.

By day 7.5 p.c., yolk cavity HA appears mainly derived from the EmEn, as this was the only adjacent tissue capable of synthesizing HA *in vitro*; amniotic HA is presumably synthesized by the surrounding EmEc, which was also capable of generating HA *in vitro*; and HA in the exocoelomic cavity probably derives from its mesodermal lining (Figs 1E, 3). The source of HA in the ectoplacental cavity (Fig. 1E) is less clear, as this cavity is solely bounded by ExEc which is HA negative *in vivo* (Fig. 1E), and was not capable of synthesizing the molecule following isolation and culture (Fig. 3). HA in the ectoplacental cavity may therefore represent secreted material from the proamniotic cavity, which becomes trapped during closure of the chorion, however, ExEc may have an HA-synthetic capability *in vivo*, possibly under the control of inductive signals from the adjacent chorionic mesoderm. Conclusive resolution of this problem awaits the development of an in

situ probe to the HA synthase molecule itself (see Prehm, 1989).

The extraembryonic membranes, and the cavities they enclose, are phylogenetically conserved structures derived from the egg integuments of early amniotes. Their principle functions in mammals are considered to be: maintenance of a physiological environment conducive to development; protection of the embryo against mechanical and chemical insult and immunological rejection; the physical separation of adjacent cell layers during differentiation; and the displacement of surrounding maternal tissues, allowing room for continued foetal growth. Expansion of these structures is achieved by the generation of a high osmotic pressure within the fluid-filled cavities. Although, on the basis of the data presented here, it is only possible to speculate as to the physiological significance of HA secretion into these cavities, it seems reasonable to expect that the exceptional hydrophilic properties of the molecule (Laurent and Ogston, 1963), and its ability to form hydrated gels at low concentrations (Comper and Laurent, 1978), would inevitably contribute to the generation of a high osmotic potential and the maintenance of mechanical rigidity during cavity expansion. Thus HA may function here in much the same way as it does in synovial fluid and vitreous humor, by acting as an organic osmolite and a 'shock absorber' (Balazs, 1965; Sundblad, 1965; Swann, 1978). This novel role for HA in promoting cavity expansion might be testable by injecting exogenous hyaluronidases into developing postimplantation embryos *in vivo* (Papaioannou, 1990), or *in vitro*, and monitoring their effect on the subsequent cavity expansion kinetics. A similar approach has been used previously, with considerable success, in evaluating the role of HA in cardiac cushion formation (Nakamura and Manasek, 1981) and the development of semicircular canals in the inner ear (Haddon and Lewis, 1991).

Sources of mesodermal HA during gastrulation and migration from the primitive streak

Early studies of HA in embryogenesis detected significant upregulation of HA synthesis during gastrulation and mesoderm migration in both chick (Manasek, 1975; Solursh, 1976) and rat (Solursh and Morriss, 1977; Fisher and Solursh, 1977) embryos; however, this is the first report to demonstrate that mesodermally associated HA may be synthesized by both EmEc and EmEn, as well as mesoderm, during gastrulation (Figs 1E, 3). Poelmann et al. (1990) found uniform extracellular HA staining in migrating rat mesoderm, as well as the basement membranes of EmEc and EmEn at this stage, but no attempt to define the cellular source of the molecule was made. It therefore appears that both EmEc and EmEn contribute to mesodermally associated HA *in vivo*, and may generate a hydrated pathway for cell migration prior to mesoderm invasion. A similar mechanism has been demonstrated previously during mesenchyme invasion of the primary corneal stroma in the developing eye (Toole and Trelstad, 1971; Meier and Hay, 1973) and during neural crest migration (Solursh et al., 1979a,b). Interestingly, as observed by Poelmann et al. (1990) in the rat, no HA was detected around cells entering the primitive streak in the mouse (Fig. 1D,E), suggesting that synthesis is only initiated by

mesoderm cells as they exit the streak in the mesodermal wings.

Role of growth factors and paracrine induction in the regulation of HA synthesis

Since HA synthesis by the HA-positive tissues was dramatically enhanced by culture in medium containing serum, compared with that obtained in medium supplemented with the ITS+TM serum substitute (Fig. 3), additional growth factors, present in serum but absent from the substitute, are clearly required for optimal HA synthesis *in vitro*, and probably also *in vivo*. The molecular mechanisms regulating the biosynthesis of HA remain to be elucidated; however, several stimulatory factors are known, including EGF, PDGF, TGF, IGF-1 and IL-1 (Laurent and Fraser, 1986; Toole et al., 1989; Heldin et al., 1989), and both tyrosine phosphorylation and protein kinase C activation are involved (Heldin et al., 1992). Isolation and culture of dissected cell lineages from the early embryo, in conjunction with histological and biochemical analyses of HA synthesis *in vitro*, should permit a more comprehensive analysis of the role of growth factors in governing HA synthesis at this crucial early stage of development.

The principle advantage of the tissue-isolation experiments described in this paper is that they provide an opportunity to investigate the synthetic profile of different embryonic cell types when the amount of experimental material is severely limited and when no RNA probes to the molecule in question, or its synthase, are available. The main disadvantage of these studies is that they may reveal developmental liabilities which are not normally expressed *in vivo*, but appear when tissue interactions are disrupted or altered (discussed by Beddington, 1986). For example, visceral extraembryonic endoderm does not synthesize -foetoprotein in the intact embryo (Dziadek and Adamson, 1978; Dziadek and Andrews, 1983), but once isolated from its underlying ExEc it initiates synthesis of the protein (Dziadek, 1978). Positional cues are obviously important in determining the secretory phenotype of the various cell types, since EmEn was able to synthesize HA during culture, whereas ExEn was not (Fig. 3). Positional information can be provided by inductive signals generated by adjacent embryonic tissues. Further studies involving the culture of tissue recombinants, as described previously (Dziadek and Adamson, 1978; Hogan and Tilly, 1981; Rossant and Tamura-Lis, 1981), will be required to evaluate the potential role of inductive interactions in regulating HA synthesis in the early mammalian embryo.

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