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MOUSE EMBRYO CULTURE SYSTEMS FOR POST-IMPLANTATION STAGE AND EXPRESSION OF LIM CLASS HOMEODOMAIN PROTEIN, LIM-1, IN EARLY MOUSE EMBRYOGENESIS

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A culture method for mouse embryos of post-implantation stage has not yet been established. In this study, we tried several methods of mouse embryo culture from blastocyst to early-somite stage using co-culture technique, human cord and/or rat sera and cytokines including leukemia inhibitory factor (LIF). Among feeder cells examined, placental cells appeared to secrete some factor(s) to promote post-implantation development. Among the sera examined, human cord serum supported best the post-implantation development. Although LIF seemed to promote pre-implantation development and implantation, it did not support post-implantation development. The present culture system is thus useful for observing embryos for two to four days after implantation. Using this system, Lim-1, a LIM class homeodomain-containing transcription factor, was observed in a part of the embryo and is suggested to play a role in early embryogenensis.

Key words: mouse embryo, culture, post-implantation stage, Lim-1

Culture procedures have been established for mouse embryos during pre-implantation stage and for early- to mid-organogenesis stage (1). These techniques have greatly advanced the study of mammalian developmental biology. However, a culture method for mouse or mammalian embryos from implantation stage through pre-organogenesis stage has not yet been established and the lack of a suitable culture method has hampered detailed analysis of many crucial events of early mammalian development. Hsu and his colleagues (2, 3) reported a procedure by which they cultured embryos from pre-implantation to organogenesis stages. However, frequency of well-developed embryos was not high.

In this study, we tried several methods of mouse embryo culture from blastocyst to early-somite stage using co-culture technique (4) and human cord and/or rat sera (5), and the results of *in vitro* development were compared with those of *in vivo* development. We also examined the effects of leukemia inhibitory factor (LIF) and granulocyte-macrophage colony stimulating factor (GM-CSF) in the culture, both of which have been shown to promote development of pre-implantation stage embryos. However, their effects on embryo development during post-implantation stage remain unclear. Amniotic fluid of chick embryo was reported to be advantageous for early embryo culture and to release the "*in vitro* two cell block", by which cultured mammalian embryos tend to arrest at the two cell stage (6). Therefore, we also investigated the effect of amniotic fluid in the culture.

Using the culture system in this study, we further examined immunohistochemically the expression pattern of LIM class homeodomain protein, Lim-1, and compared it with the *in vivo* expression pattern. The ortholog of this transcriptional factor in *Xenopus* has been shown to play important roles in early embryogenesis (7, 8) and the role in organogenesis in mice has also been documented from the knockout mouse study (9, 10). However, the expression and function remain unclear in mouse early embryos, in which interactions with maternal tissues may cause significant differences from *Xenopus.*

MATERIALS AND METHODS

Jcl:ICR mice of 8-15 weeks old were used.

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Females were mated with males after hormonally-induced estrus, and blastocysts were harvested in the afternoon of day 3 of gestation (E3). Embryos were cultured in CMRL1066 medium supplemented with 1 mM L-glutamine and 1 mM sodium pyruvate at 37.5℃ under 5% CO_2 and 95% air. Sera added and the time course of development of successfully cultured embryos are shown in Table 1. Falcon collagen type I-coated dishes were used (Becton Dickinson and Company, Plymouth, UK). Murine recombinant LIF 5 ng/ml (Funakoshi, Tokyo, Japan), murine recombinant GM-CSF 5 ng/ml (Funakoshi, Tokyo, Japan), and amniotic fluid which was collected from 10 days old chick embryos (10% (v/v) final concentration) were added to the medium as indicated. General experimental schedule is shown in Fig. 1.

Table 1. Time course of mouse embryo culture and sera used

Gestationl stage (day)	Developmental events	Culture period (day)	Serum	Agitation
3.5		0	FBS 10%	
4.0		1		
4.5		2	FBS 10%	
5.0	Formation of the bilaminar germ disc	3	FBS 10% + HCS 10% (RS 20%)	
5.5		4	HCS 30% (RS 40%)	+
6.0				
6.5	Egg cylinder	5	HCS 40% (RS 50%)	+
7.0	Primitive streak	6	HCS 40% + RS 10%	+
7.5	Neurula	7	RS 100%	+
8.0	Somite 1-4	8		+

FBS: fetal bovine serum
HCS: human cord serum
RS: rat serum
RS in parenthesis was used when HCS was not used.

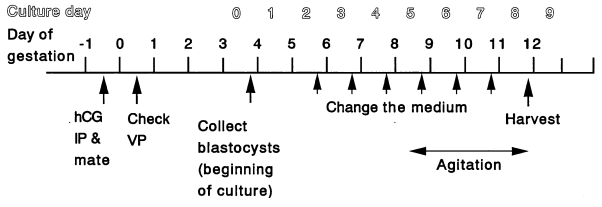


Fig. 1. General experimental schedule.

As feeder layers, primary cultured cells of mouse placenta and endometrium were used. Placentae were collected from E12 or E13 pregnant mice, minced and digested in 0.25% trypsin and 0.1% EDTA for 15 min. After filtration with a metalic filter (120 mesh/mm), cell suspension was inoculated into dishes at a rate of 10^6/ml. For the primary culture of endometrium, pregnant mouse uteri at E3 were collected, cut into pieces, and digested in 0.25% trypsin and 0.1% EDTA for 30 min. Cell were filtered with a 200-mesh metalic filter and seeded on dishes at 10^6/ml. After 48 h incubation and washing out of blood cells and free cells, these primary cultures were used as feeder layers.

We observed intermittently *in vitro* development of embryos, which were cultured most successfully with human cord serum (HCS), using a laser video disc recording system (LVR3000AN, SONY), and compared the development of cultured embryos with that of embryos developed *in vivo*, in which fertilization time was strictly determined by monitoring the mating time to standardize the development.

Whole mount immunohistochemistry for Lim-1 was performed as previously reported (7, 11). Briefly, embryos were fixed in MEMFA (100 mM MOPS, 1 mM $MgSO_4$, 2 mM EGTA, 3.8% formaldehyde). Anti-Xlim-1 antibody (kindly gifted from Dr. A.A. Karavanov) is a polyclonal rabbit antibody produced against the fusion of GST to the C-terminal part of Xlim-1 protein and has been shown to crossreact with Lim-1 protein in other vertebrates (11). Anti-Xlim-1 antibody was diluted 1/200 in Boehringer-Mannheim blocking reagent and applied to specimens overnight at 4℃. Following washes, specimens were treated with secondary antibody conjugated to alkaline phosphatase (Boehringer-Mannheim) and chromogenic reaction was done according to the manufacturer's protocol. Control reactions were performed without the primary antibody.

RESULTS AND DISCUSSION

Evaluation of culture systems

1) Effects of feeder cells and human cord and rat sera

Embryos were co-cultured on the placental or endometrial cells as feeder layers, or on collagen-coated dishes. The frequency of embryos which developed to the primitive streak stage on the

placental cells (3.8%, data not shown) tended to be higher than those on collagen dishes (2.6%, group A in Table 2) and those on the endometrial cells (0%) (data not shown). Our results are consistent with the previous report that decidual cells accelerated the attachment of hatched blastocysts (4).

Next, we cultured embryos on pored membrane (12 μm) in order to separate embryos from the feeder cells, since embryos together with the feeder cells often detached from the dish during the culture in the pilot experiments. When the placental cells were used as the feeder cells, the ratio of embryos which developed to the primitive streak stage to the total embryos cultured on the membrane (5.2%) was nearly the same with that of embryos cultured directly on the placental cells (group C in Table 2). Further, when conditioned medium from the placental cell culture was used on collagen-coated dishes without feeder cells, the ratio of primitive streak stage embryos (6.2%, group D in Table 2) was nearly the same with those of co-culture group (3.8%) and membrane group (5.2%) using placental cells as feeder cells and tended to be higher than those without placental cells, albeit without statistically significant difference (Table 2). It is thus suggested that some factor(s) which placental cells secrete promote development of mouse embryos after implantation.

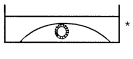
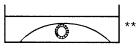
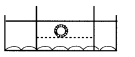
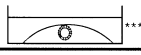
In the medium using HCS and rat serum, together with a gentle agitation for a better exposure of the embryos to the medium (Table 1, group B in Table 2), the ratio of embryos which developed to primitive streak stage (15.7%) and that of heart beating embryos (2.5%) (Figs. 2, 3) were significantly higher than those of embryos developed in the co-culture system with placental cells as a feeder layer (5.2% and 0.6%, respectively, Table 2), or in the medium without HCS (data not shown).

2) Effects of LIF, GM-CSF and amniotic fluid

We next cultured embryos in medium supplemented with LIF, GM-CSF and/or amniotic fluid on collagen-coated dishes during pre- and post-implantation periods. All of these factors and solution have been shown to promote development of pre-implantation stage embryos. In mice, mRNA of LIF is expressed in the endometrial glands of the uterus coincidently with the blastocyst implantation (12). Synthesis of GM-CSF is maintained at a high level during the pre-implantation period and estrus and its mRNA is expressed in the decidua (13). However, their effects on embryo development during post-implantation stage have not been systematically examined.

During pre-implantation period, LIF and amniotic fluid promoted blastocysts to attach on culture dishes (Fig. 4). However, mixture of these cytokines decreased promoting effect of each cytokine (data not shown). LIF receptors have been found on the expanded blastocyst, therefore LIF may regulate growth and implantation of blastocysts (14). During the present post-implantation culture, in the medium supplemented with LIF, the inner cell mass proliferated for four days, then it stopped proliferation and embryos did not further differentiate (data not shown). *In vivo*, LIF transcripts were detected in the decidual swelling of the endometrial gland cells surrounding the newly implanted embryo (15). On the other hand, LIF has differentiation inhibiting effect on embryonic stem (ES) cells (16) and it is generally used to maintain ES cells in an undifferentiated state. Therefore, although LIF appears to promote proliferation without prominent embryonic differentiation and support implantation, it may not further promote proliferation with embryonic differentiation from the post-implantation stage.

Table 2. Development of embryos cultured with human cord serum, the feeder layer of the placental cells or the conditioned medium from the placental cell culture

Group	Culture methods	Number of blastocysts	Number of heart beating embryos (%)	Number of primitive streak stage embryos (%)	
A	 *	39	0	1 (2.6)	
B	 **	362	9 (2.5)	57 (15.7)	+
C		174	1 (0.6)	9 (5.2)	+
D	 ***	80	0	5 (6.2)	

*: Embryos were cultured in the medium using only rat serum.

**: Embryos were cultured in the medium using human cord serum and rat serum.

***: Embryos were cultured in the conditioned medium from the placental cell culture.

+: $P<0.001$ (χ^2 test)

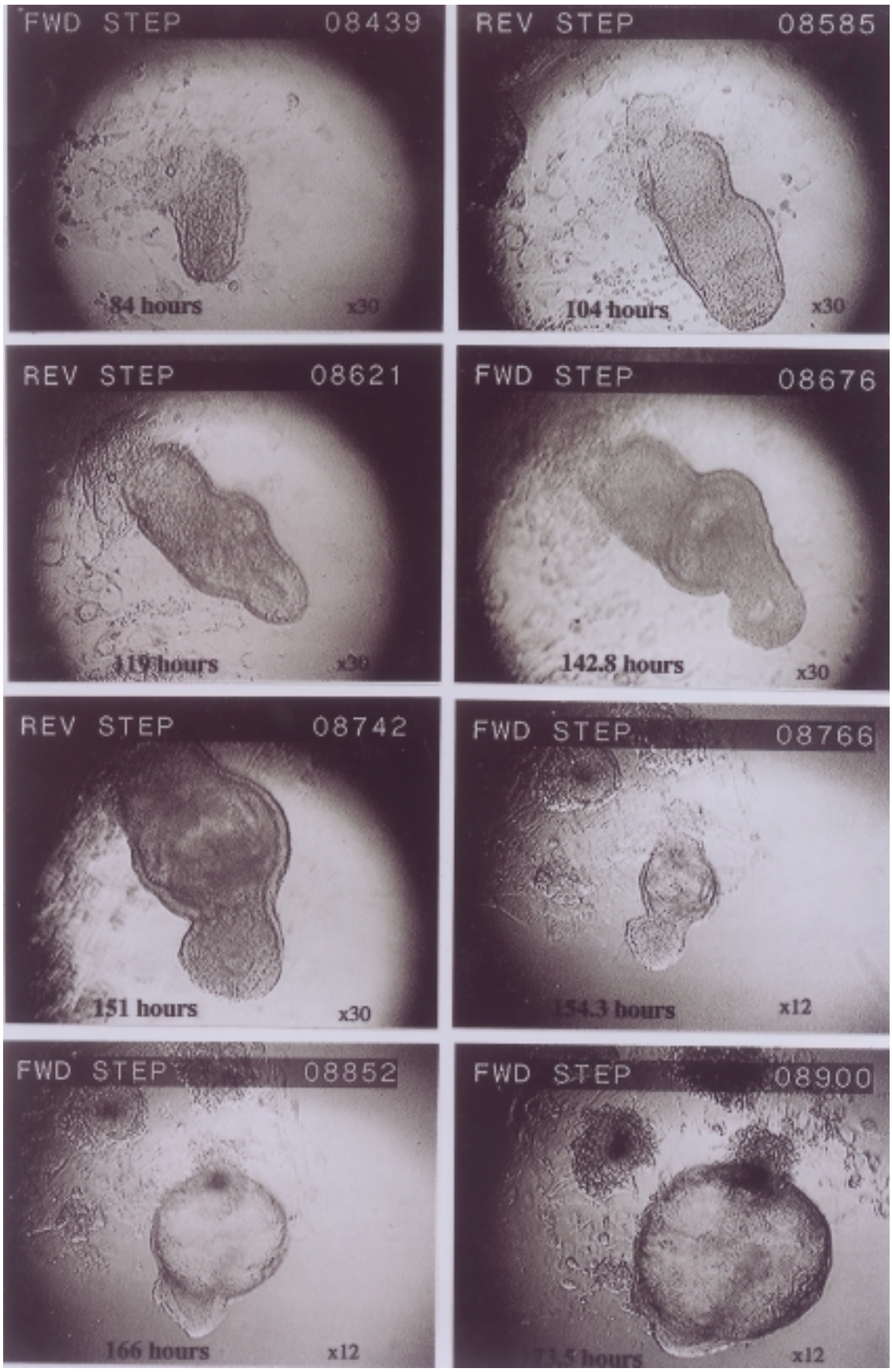


Fig. 2. *In vitro* development to the heart-beating stage of a mouse embryo recorded by the video disc record ing system (see Fig. 3). Hours of culture and magnifications are indicated in each panel. Numbers at the right-top corner are the serial number of the record.

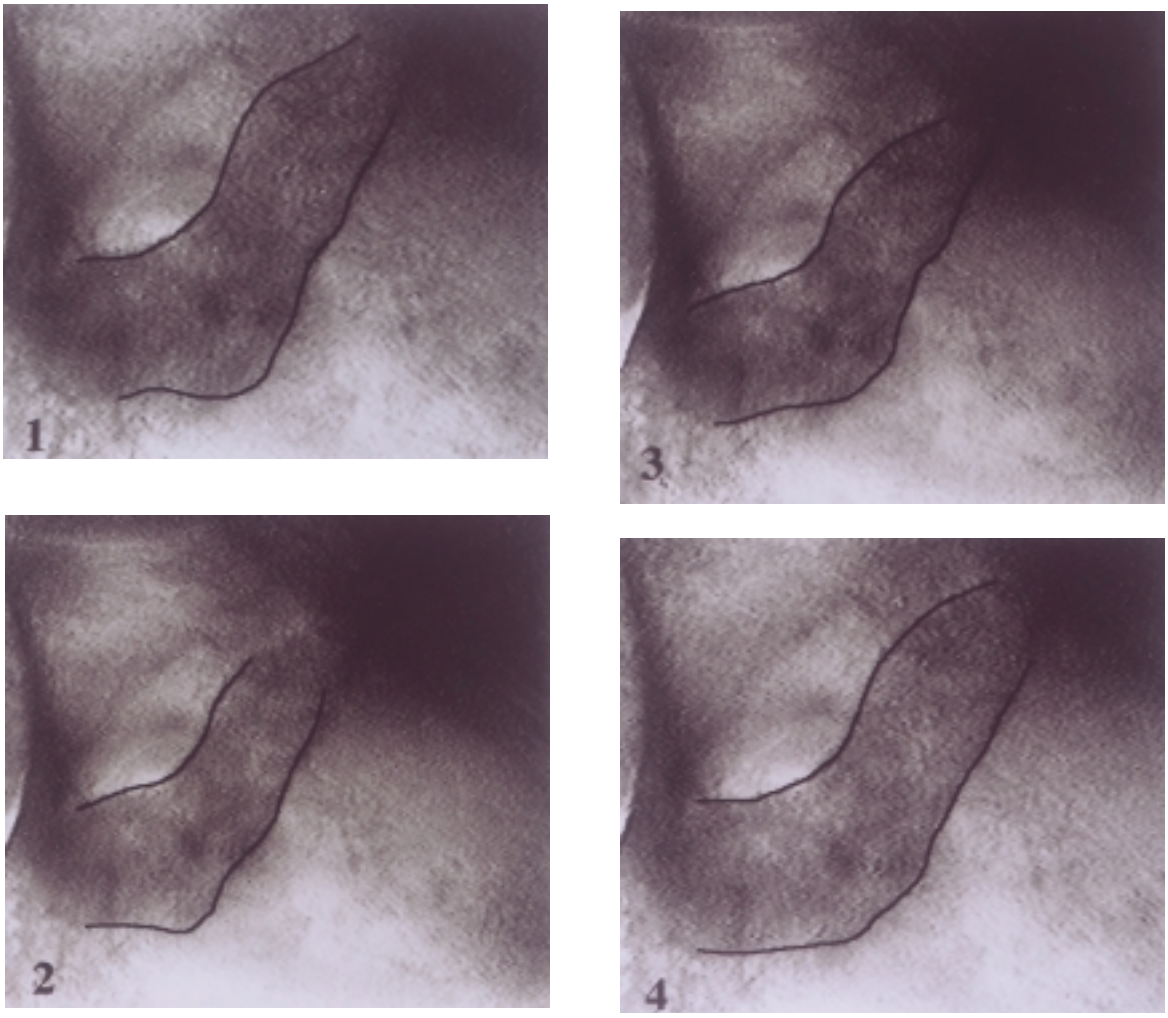


Fig. 3. The beating heart of the embryo shown in Fig. 2. Panels numbered one through four are a series of record, and the outlines of the heart tube are marked on the images. Note that panels 1 and 4 are diastolic and panels 2 and 3 are systolic.

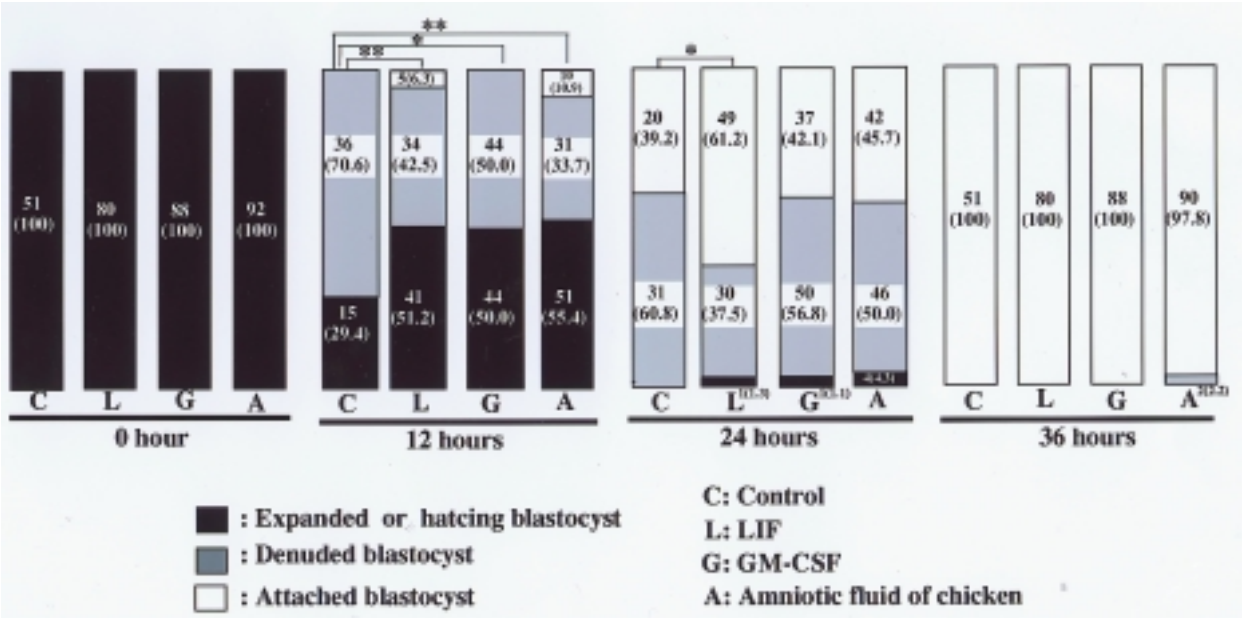


Fig 4. Effects of LIF, GM-CSF and amniotic fluid of chicken on pre-implantation development of mouse embryos. Numbers of embryos and their percentiles are indicated in columns. **: $P<0.05$, *: $P<0.01$ (Mann-Whitney's U-test).

Stimulation of proliferation of the trophoblasts in the ectoplacental cone was reported in the presence of GM-CSF *in vitro* (17). However, in the present study, the embryos cultured in the GM-CSF medium developed similarly to those in the cytokine-free medium, therefore we could not detect significant effects of GM-CSF during post-implantation (data not shown). In media containing LIF and GM-CSF, embryos slightly developed but they could not form egg cylinder (data not shown).

Comparison of between *in vitro* and *in vivo* embryo development

In the comparative observation between *in vitro* and *in vivo* development (Table 1, Fig. 2), the cultured embryos were approximately 1.5 days behind *in vivo* embryos to develop to egg cylinder stage, and 2.0 days behind those *in vivo* to reach primitive streak stage after fertilization. Thus, the present culture system is useful for observing mouse embryos for up to the stage of two to four days after implantation, but not further later.

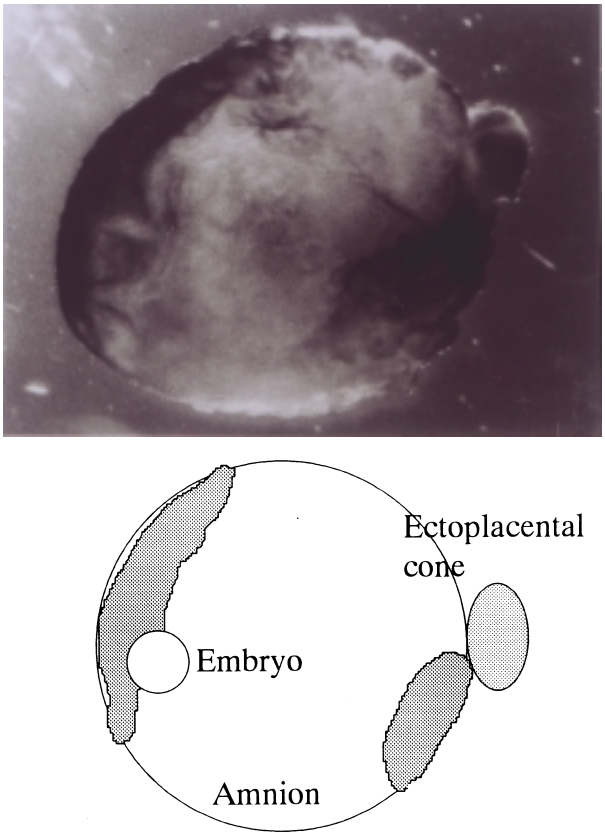


Fig. 5. (Top) Lim-1 expression in an 8-day cultured embryo as revealed by immunohistochemistry. See text for the expression pattern. (Bottom) Schematic representation of the top panel.

Expression of Lim-1 in early mouse embryos

We performed immunohistochemistry of Lim-1, the product of a murine LIM class homeobox gene *lim-1* in embryos developed *in vivo* and *in vitro* to compare the expression patterns. Lim-1 was detected in the inner cell mass and trophoblasts of the embryos cultured for 72 hours and similarly in the corresponding E5.5 embryos developed *in vivo* (data not shown). Lim-1 was localized in a hemisphere of the yolk sack and in the ectoplacental cone of the embryos cultured for 192 hours (Fig. 5) and was localized in a part of the yolk sack of E7.5 *in vivo* embryos. These results suggest that Lim-1 is involved in early embryogenesis in mammals. However, to determine the precise function and whether there is significant difference in the expression pattern between *in vitro* and *in vivo*, further detailed analysis is necessary.

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