Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4756–4760, June 1990
Developmental Biology

Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors

(morula/blastocyst/epidermal growth factor receptor/autocrine/paracrine)

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Communicated by Clement L. Markert, April 13, 1990

ABSTRACT **We have established a model that shows cooperative interaction among preimplantation embryos and the role of growth factors on their development and growth. Two-cell mouse embryos cultured singly in 25-μl microdrops had inferior development to blastocysts and lower cell numbers per blastocyst compared with those cultured in groups of 5 or 10. The inferior development of singly cultured embryos was markedly improved by addition of epidermal growth factor (EGF) or transforming growth factor α or β1 (TGF-α or TGF-β1) to the culture medium. The stage of embryonic development, primarily affected by these treatments, was between eight-cell/morula and blastocyst. Furthermore, blastocysts developed from eight-cell embryos cultured in groups or singly in the presence of EGF showed a higher incidence of zona hatching compared with those cultured singly in the absence of EGF. Detection of EGF receptors on the embryonic cell surface at eight-cell/morula and blastocyst stages suggests beneficial effects of EGF or TGF-α on preimplantation embryo development and blastocyst functions. Insulin-like growth factor I (IGF-I) had no influence on embryo development. To further document the cooperative interactions among embryos, the volume of the culture medium was doubled to 50 μl. This increase in culture volume was even more detrimental to the development of singly cultured embryos. However, this detrimental effect was significantly reversed by EGF and reversed even more markedly by a combination of EGF and TGF-β1 but not by TGF-β1 alone. Although TGF-β1 plus IGF-I caused a modest improvement of embryo development, the response was not as great as shown by EGF alone. Furthermore, IGF-I had no additive effect on EGF-induced embryonic development. The study presents clear evidence that specific growth factors of embryonic and/or reproductive tract origin participate in preimplantation embryo development and blastocyst functions in an autocrine/paracrine manner.**

The development of mouse preimplantation embryos to the blastocyst stage is, at least partially, a function of autoregulation. Two distinct features of the preimplantation embryo development are activation and cleavage of the fertilized egg and differentiation of the embryonic cells into inner cell mass (ICM) and trophectoderm at the blastocyst stage. The mechanism by which proliferation and differentiation are controlled in the preimplantation embryo is unclear. Although fertilized eggs can develop into blastocysts within the reproductive tract of steroid hormone-depleted mice, a substantial loss in the number of embryos and cells per embryo occurs under this condition. Treatment with an appropriate combination of progesterone (P_4) and estrogen (E) reverses these defects (1, 2). These observations suggest that although certain factors of embryonic origin participate in an autocrine regulation of embryonic development, the full complement of preimplantation embryo development requires additional paracrine factors that originate from the reproductive tract under the influence of P_4 and E. There is no convincing evidence for direct effects of P_4 and/or E on preimplantation embryo development. The concept of autocrine regulation of preimplantation embryo development and differentiation is further supported by the fact that preimplantation embryos can develop into blastocysts *in vitro* in a simple medium (3). However, embryonic growth rate is slower *in vitro* and there are fewer cells in *in vitro*-grown blastocysts (4). This suggests that absence of growth factors of reproductive tract origin and/or dilution of these factors released by the embryos in the culture medium are responsible for retarded development of preimplantation embryos *in vitro*. This contention is further supported by recent reports of synthesis of several growth factors by the preimplantation embryo (5) and the uterus (6–8). Thus growth factors produced by the preimplantation embryo and/or the reproductive tract are available to influence embryonic development and function in an autocrine/paracrine manner. If growth factors produced by the preimplantation embryo act on them, then it can be postulated that preimplantation embryos cultured in a small number in a defined volume of culture medium will show inferior development compared with those cultured as a large group. This could reflect a greater dilution of the growth factors secreted from a small number of cultured embryos into the medium compared with those cocultured in a larger group. This inferior development should then be corrected by addition of growth factors in the culture medium. To test this hypothesis, we have developed a unique model to examine the cooperative interaction among embryos and the role of growth factors and their interactions on preimplantation embryo development *in vitro*.

MATERIALS AND METHODS

Growth Factors and Other Reagents. Receptor-grade mouse submandibular gland epidermal growth factor (EGF) was purchased from Sigma. Human platelet-derived transforming growth factor β1 (TGF-β1) was provided by Anita Roberts (National Institutes of Health) and recombinant human transforming growth factor α (TGF-α) and insulin-like growth factor I (IGF-I) were purchased from Bachem and Amgen Biologicals, respectively. Highly purified ^{125}I-labeled EGF (^{125}I-EGF) (mouse submandibular gland) was provided by Amal Mukherjee (Diagnostic Systems Laboratories, Webster, TX). Crystalline bovine serum albumin was purchased from Sigma (A-4378). As determined by agarose gel electrophoresis, the purity of this bovine serum albumin is 98%; the remainder is globulins. All other culture-grade reagents were obtained from Sigma, Fisher, or Mallinckrodt.

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Abbreviations: EGF, epidermal growth factor; TGF, transforming growth factor; IGF, insulin-like growth factor; ICM, inner cell mass.
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Animals. Female mice (20–25 g) of Charles River strain (CD-1) were mated with the males of the same strain. The morning of finding the vaginal plug was designated as day 1 of pregnancy.

Embryo Culture. To study cooperative interactions among embryos and the role of growth factors on preimplantation embryo development, two-cell embryos on day 2 (0830–0900 hr) were recovered and pooled from several mice in Whitten's medium containing 0.3% bovine serum albumin (9). They were washed four times in the same medium. Embryos were cultured in groups of 5 or 10 or singly in microdrops (25 or 50 μl) of Whitten's medium (9, 10) under silicon oil in an atmosphere of 5% CO_2/95% air at 37°C for 72 hr in the presence or absence of various concentrations of specific growth factors or their combinations. The growth factors were added at the beginning of cultures. The embryos were observed every 24 hr to monitor their development. At the termination of culture, cell number per blastocyst was determined (11). To study cooperative interactions among embryos and the role of growth factors on zona hatching *in vitro* (complete escape of the blastocyst from its zona pellucida), eight-cell embryos recovered on day 3 (0830–0900 hr) were cultured in groups of 5–14 or singly in the presence or absence of EGF for 72 hr. The embryos were examined every 12 hr for their development. Each experiment was repeated three to nine times with the exception of numerous replicates of controls included in each experimental repetition.

EGF Binding Analysis. Preimplantation embryos at one-cell, two-cell, four-cell, eight-cell, morula, and blastocyst stages were recovered in Whitten's medium on days 1–4 of pregnancy. They were freed of zona pellucidae by a brief exposure to 0.5% Pronase solution in phosphate-buffered saline (PBS), washed several times, and cultured for 2 hr in the same medium. They were then incubated in 25-μl microdrops under silicon oil with 20 pM ^{125}I-EGF (specific activity, 150 ± 10 μCi/μg; 1 Ci = 37 GBq) in PBS containing 0.1% bovine serum albumin in the presence or absence of a 500-fold excess of unlabeled EGF, TGF-α, TGF-β1, or IGF-I for 30 min at 37°C. After incubation, embryos were washed five times in cold (4°C) PBS and fixed in cold 4% paraformaldehyde in PBS for 15 min. They were then "cytospun" onto poly(L-lysine)-coated slides. The slides were dehydrated through ascending grades of alcohol, dipped into Kodak NBT-2 emulsion, exposed for 7 days, developed, and stained in hematoxylin.

RESULTS

As shown in Fig. 1, only 49% of the embryos cultured singly in 25 μl of medium developed to blastocysts compared with >80% of those cultured in groups. This inferior development of singly cultured embryos to blastocysts was markedly improved in a concentration-dependent manner by the addition of EGF or TGF-α (Fig. 2). Furthermore, although none of the embryos cultured singly formed blastocysts at 48 hr of culture, about 20% of the embryos cultured in groups or about 40% of the embryos cultured singly in the presence of EGF (10 ng/ml) developed to blastocysts by this time (data not shown). EGF at 200 ng/ml did not further improve embryo development compared with that at 10 ng/ml, and this growth factor had little effect on development when the embryos were cultured in groups (data not shown). The developmental arrest of embryos cultured singly was primarily at the eight-cell/morula stage. Although IGF-I was ineffective in improving embryo development to blastocysts, TGF-β1 was very effective in this response (Fig. 2). As shown in Figs. 1 and 3, fewer numbers of cells per blastocyst (34.0 ± 1.5) of the singly cultured embryos almost doubled when the embryos were cultured in groups or singly in the presence of EGF. Although TGF-α significantly increased

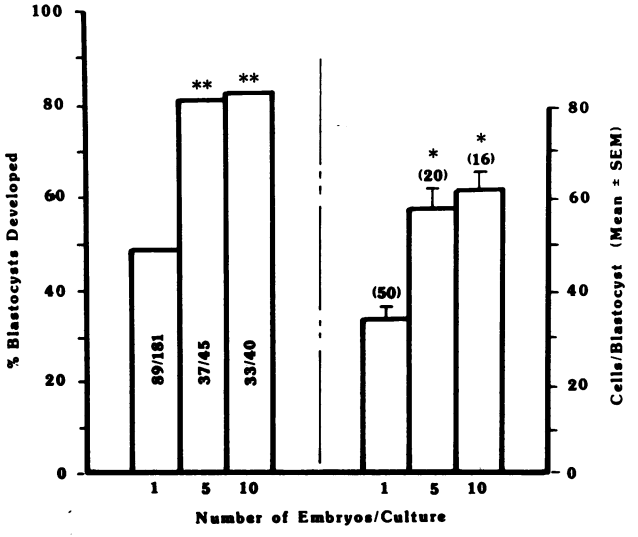

FIG. 1. Development of blastocysts and cell numbers per blastocyst following culture of two-cell embryos singly or in groups in 25 μl of medium for 72 hr. Numbers within bars indicate the number of blastocysts developed/the number of embryos cultured. Numbers in parentheses indicate the number of blastocysts examined for cell count. * and ** indicate $P < 0.05$ and $P < 0.001$ (χ^2 and Scheffe's post hoc tests), respectively, compared with singly cultured embryos.

cell numbers per blastocyst, this growth factor was not as effective as EGF. Neither TGF-β1 nor IGF-I was effective in increasing cell numbers (Fig. 3). Blastocysts developed from two-cell embryos cultured in groups or singly in the presence of EGF had comparable cell numbers (61.7 ± 4.4 for 10 embryos per group and 62.7 ± 5.7 for 4 ng/ml, respectively) compared with those of *in vivo* blastocysts (62.8 ± 1.6, n = 16) recovered in the afternoon (1700 hr) of day 4 prior to implantation. On the other hand, *in vivo* blastocysts recovered on the morning of day 4 (0800 hr) had cell numbers (34.3 ± 3.0, n = 11) that were very close to those of singly cultured embryos in the absence of any growth factors as shown above. Blastocysts developed from singly cultured embryos in the presence of EGF (4 ng/ml) or TGF-β1 (2 ng/ml) were physiologically normal, as evident by delivery of healthy offspring following their transfer to foster mothers (EGF:

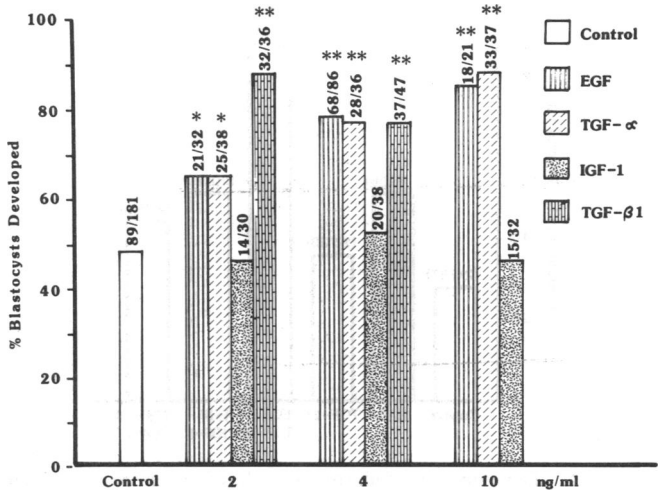

FIG. 2. Development of blastocysts following culture of two-cell embryos singly in 25 μl of medium for 72 hr in the absence or presence of specific growth factors. Number on each bar indicate the number of blastocysts developed/the number of embryos cultured. * and ** indicate $P < 0.05$ and $P < 0.001$ (χ^2 test), respectively, compared with singly cultured embryos (Control).

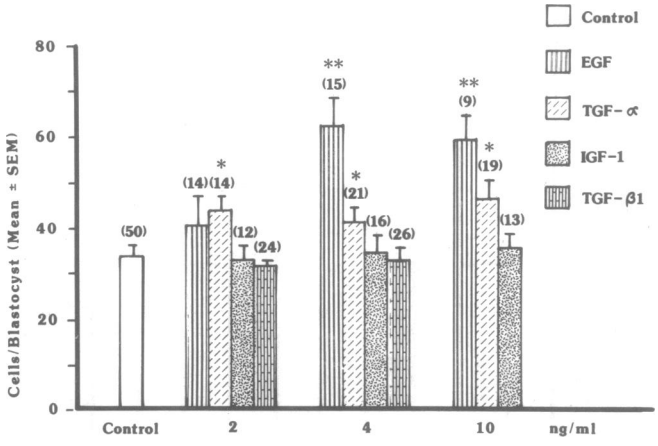

FIG. 3. Number of cells (mean ± SEM) per blastocyst developed from two-cell embryos cultured singly in 25 μl of medium for 72 hr in the absence or presence of specific growth factors. Numbers in parentheses indicate the number of blastocysts examined for cell count. * and ** indicate $P < 0.05$ and $P < 0.01$ (Scheffe's post hoc test), respectively, compared with singly cultured embryos (Control).

6/10 and 5/11; TGF-β1: 7/12 and 5/10). However, because of the complex nature and number of experiments needed to obtain meaningful information, we do not know at this time the differences in the pregnancy outcome of transferred embryos cultured in groups or singly in the presence or absence of growth factors.

Doubling of the volume of culture medium from 25 μl to 50 μl further decreased the number of blastocysts developed from singly cultured embryos (49% vs. 28%) (Fig. 1 vs. Fig. 4). Although addition of EGF significantly improved embryonic development to blastocysts (51%), further improvement was noted (87%) when a combination of EGF and TGF-βI was added in the culture medium (Fig. 4). TGF-β1 alone was not effective in improving embryo development. TGF-β1 plus IGF-I showed a modest improvement in embryo development, but the response was not any better than that achieved by EGF alone. Furthermore, IGF-I did not influence EGF-induced embryonic development (Fig. 4). These growth factors singly or in combination were ineffective in

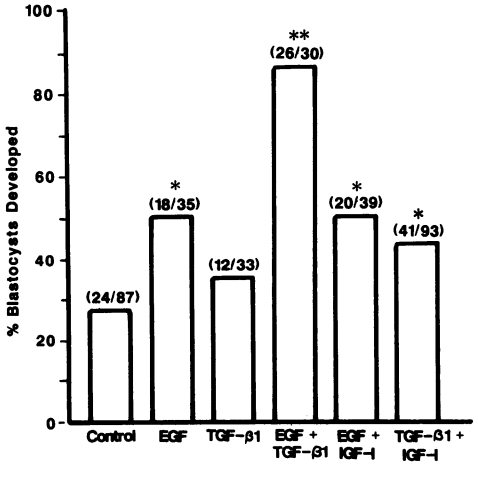

FIG. 4. Development of blastocysts following culture of two-cell embryos singly in 50 μl of medium for 72 hr in the absence or presence of specific growth factors alone or their combinations. EGF was used at 4 ng/ml; other growth factors were used at 2 ng/ml. Numbers within parentheses indicate the number of blastocysts developed/the number of embryos cultured. * and ** indicate $P < 0.05$ and $P < 0.001$ (χ^2 test), respectively, compared with singly cultured embryos (Control).

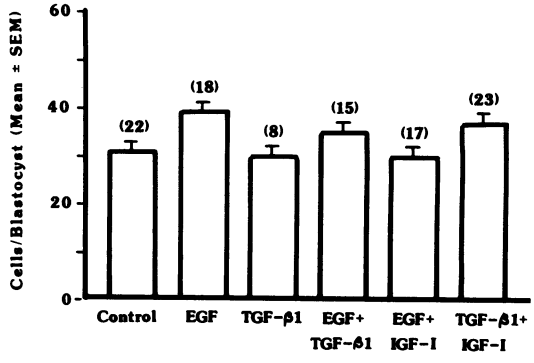

FIG. 5. Number of cells (mean ± SEM) per blastocyst developed from two-cell embryos cultured singly in 50 μl of medium for 72 hr in the presence or absence of specific growth factors alone or their combinations. Concentrations of growth factors used were as in Fig. 4. Numbers within parentheses represent the number of blastocysts examined for cell count. No significant differences were observed between embryos cultured in the absence (Control) or presence of the growth factors (Scheffe's posthoc test).

increasing cell numbers per blastocyst of singly cultured embryos in 50 μl of medium compared with those cultured in 25 μl of medium (Fig. 5).

Table 1 shows that although most of the eight-cell embryos cultured in groups or singly in the presence or absence of EGF developed to blastocysts, the singly cultured blastocysts showed a poor incidence of zona hatching (11%) compared with those cultured in groups (52%). The poor rate of zona hatching of singly cultured blastocysts was remarkably improved in the presence of EGF in a concentration-dependent manner. However, the rate of zona hatching of blastocysts cultured in groups was only modestly improved by EGF (52% vs. 64%). Although a large number of eight-cell embryos cultured singly in 25 μl of medium developed to blastocysts by 32 hr in the absence (86%, $n = 35$) or presence (95%, $n = 43$) of EGF, the cell number per blastocyst increased significantly ($P < 0.0001$) in the presence of EGF (43.1 ± 1.7, $n = 30$) in contrast to that in the absence of this growth factor (31.9 ± 1.7, $n = 24$).

Under our experimental conditions, low-intensity specific autoradiographic signals for ^{125}I-EGF binding to the embryonic cell surface were first detected at the eight-cell stage (data not shown). The binding then increased at the morula and blastocyst stages (Fig. 6). The binding was specific since unlabeled EGF or TGF-α, but not IGF-1 or TGF-β1, successfully competed to displace the binding of ^{125}I-EGF. In the

Table 1. Effect of EGF on blastocyst hatching *in vitro* following culture of eight-cell embryos in 25 μl of medium for 72 hr

EGF, ng/ml	Embryos cultured, no.	Embryos developed to blastocysts		Hatched blastocysts	
		No.	%	No.	%
Embryos cultured singly					
0	32	28	87.5	3	10.7
4	28	24	85.7	9	37.5*
10	33	31	93.9	20	64.5†
Embryos cultured in group					
0	85	75	88.2	39	52.0†
10	73	66	90.4	42	63.6†

For group culture, 5–14 embryos were used per culture.
*$P < 0.05$ (χ^2 test) compared with singly cultured embryos in the absence of EGF.
†$P < 0.0001$ (χ^2 test) compared with singly cultured embryos in the absence of EGF.

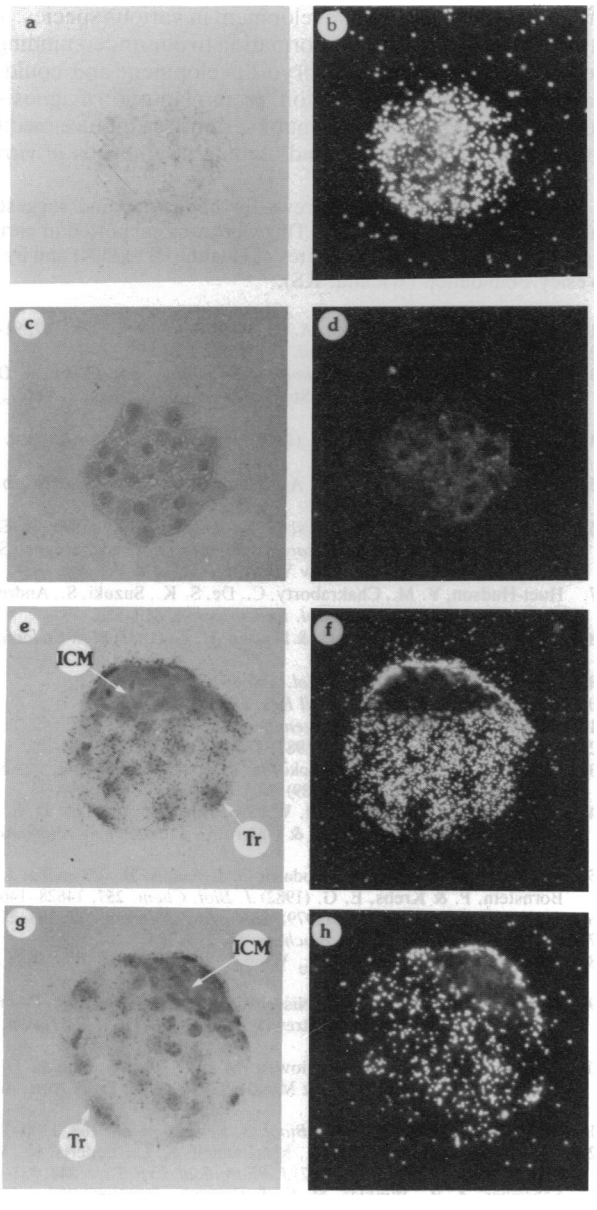

FIG. 6. Autoradiographic localization of ^{125}I-EGF binding in the morula and blastocyst. (×200.) Binding in a morula is shown in bright (*a*) and dark fields (*b*); nonspecific binding is shown in bright (*c*) and dark fields (*d*). Localization of binding in a blastocyst is shown in bright (*e*) and dark fields (*f*); nonspecific binding is shown in bright (*g*) and dark fields (*h*). Tr, trophectoderm.

blastocyst, the binding was limited to trophectoderm but not to the ICM cells.

DISCUSSION

The major finding of the present investigation is that there is a cooperative interaction among preimplantation embryos *in vitro* and that this interaction is mediated by specific growth factors released by them. This observation is important since TGF-β1 is expressed in the mouse embryo from two-cell stage, and platelet-derived growth factor A chain and TGF-α are expressed in the morula and blastocyst (5). This and our present finding suggest that preimplantation embryos are capable of promoting their own development if they are allowed to develop close to each other. However, embryo distribution in the reproductive tract may not allow them to have this privilege, and growth factors released by them may have rapid turnover *in vivo*. Thus, growth factors of reproductive tract origin are likely to complement those produced by the embryo for their continued and successful development *in vivo*. Although coculture of preimplantation embryos with oviductal cells is beneficial for preimplantation embryo development (12, 13), it is not known whether growth factors are produced by the oviduct. On the other hand, the uterus produces several growth factors, such as EGF (6, 7), TGF-α, TGF-β1, and IGF-I (our unpublished results), during the preimplantation period in a cell type-specific manner. Particularly important is our finding of immunoreactive EGF at the apical border of the luminal epithelium early on day 4 of pregnancy (6). Since embryos at the morula stage enter into the uterus late on day 3 or early on day 4, and since EGF or TGF-α mediates its functions through EGF receptors (14, 15), we suggest that TGF-α produced by the embryo (5) participates in morula to blastocyst transformation, zona shedding, and blastocyst activation in an autocrine manner, whereas EGF at the apical border of the luminal epithelium on day 4 (6) does so in a paracrine fashion.

EGF possesses well-documented mitogenic and differentiating effects (16). EGF-induced increases in cell number per blastocyst and in the number of blastocysts developed from two-cell embryos cultured singly in 25 μl of medium reflect mitogenic and differentiating effects on preimplantation development. Although TGF-α belongs to the EGF family and has 40% sequence homology with EGF (17), it was not as effective as EGF in increasing the blastocyst cell numbers. This is not surprising, since EGF and TGF-α bind differently to EGF receptors (18). This may explain differences in the potency of expressing biological activities of these two related growth factors (19–23). The failure of other investigators to clearly demonstrate the effects of these growth factors on morphologic and mitogenic development of the preimplantation mouse embryos could be due to the fact that they cultured embryos in groups (24, 25). However, one laboratory observed a moderate, but statistically significant, increase in protein synthesis in cultured morulae and blastocysts exposed to EGF (25). By the use of immunosurgery, these investigators also noted that the effect of EGF was confined to trophectoderm cells and suggested that EGF receptors are expressed in trophectoderm cells of the blastocyst during its formation from a morula. Our success in showing dramatic biological effects of EGF and specific autoradiographic localization of ^{125}I-EGF binding on embryonic cell surface at the eight-cell/morula and blastocyst stages, but not at earlier embryonic stages, provides direct evidence for the expression of a functional ligand-receptor circuit at a critical stage of embryo development and blastocyst function—i.e., morula to blastocyst transformation and zona shedding. The ICM cells did not show any autoradiographic localization of ^{125}I-EGF binding. Although the absence of EGF binding to ICM cells could be due to the inaccessibility of EGF to the ICM owing to the tight junctions in the trophectoderm, this is unlikely because immunoreactive EGF receptor protein was exclusively localized in the trophectoderm cells, and not in the ICM cells, of the day-5 postimplantation blastocyst sections (26). The presence of EGF receptors on trophoblast outgrowths and their absence on the ICM-derived cells further suggest that these receptors are expressed in the trophectoderm cells (27). This implies that EGF or TGF-α is important in promoting growth and differentiation of trophectoderm cells which proliferate at a faster rate than ICM cells (28).

TGF-β belongs to a large gene family and multiple forms of TGF-β with diverse functions have been identified (29). Because of its role in cell migration, growth, differentiation, and formation of extracellular matrix and cell surface molecules, TGF-β has been implicated in embryogenesis (29). The expression of TGF-β in tissues undergoing morphogenesis suggests its role in differentiation (29). The differential effects of this growth factor on undifferentiated and differentiated

embryonic carcinoma cell lines, used as an experimental model of early embryogenesis, also indicate that TGF-β plays an important role in early mammalian development (30). Our findings of TGF-β1's influence in improving the embryonic development to blastocysts without increasing their cell number suggest that this growth factor participates in differentiation of the preimplantation embryo. This is not surprising since TGF-β1 plus fibroblast growth factor, or TGF-β2 alone, are important morphogens for mesoderm induction in frog blastulae (31, 32). Future study will determine at what stage of development the receptors for TGF-β appear on embryonic cell surface. Our results demonstrate that the effects of EGF, TGF-α, or TGF-β1 are specific, since another mitogenic growth factor, IGF-I, had little effect on preimplantation embryo development in spite of the presence of receptors for IGF-I and insulin in the morula and blastocyst (33, 34). However, IGF-I may have other functions or could be important for preimplantation embryo development in combination with other growth factors not examined in this study. Insulin has been shown to stimulate RNA and DNA synthesis in the morula and blastocyst (33, 34). However, the superior development of embryos cultured in groups or singly in the presence of specific growth factors is not likely to be mediated by insulin, since insulin mRNA is not expressed in the preimplantation embryo (35).

Our finding of further deterioration of development of singly cultured embryos in a larger volume of culture medium (50 μl) again supports the concept that cooperative interaction among preimplantation embryos is mediated by growth factors released by them. However, the requirement for a combination of growth factors, but not specific growth factor alone, in promoting blastocyst formation and particularly their ineffectiveness in increasing the number of cells per blastocyst indicate that an additional important factor(s) was released by preimplantation embryos that became too diluted to be effective when they were cultured in 50 μl instead of 25 μl of medium.

The mechanisms by which mitogenic and differentiating effects of EGF or TGF-α and the differentiating effect of TGF-β1 on preimplantation embryos are mediated are unclear. It is possible that the mitogenic effect of EGF or TGF-α is mediated by the expression of a protooncogene, c-*myc* (36). We have recently observed that the immunoreactive c-*myc* protein first appears in the nuclei of eight-cell mouse embryos (unpublished results). Because EGF can activate plasma membrane-bound Na^+/H^+ exchanger (37, 38), the formation of blastocoel, which requires an active Na^+/H^+ exchanger (38), may be mediated by this or its related growth factor, TGF-α. The role of TGF-β1 in blastocyst formation could be mediated by its effects on differentiation of embryonic cells to form trophectoderm and establishment of tight junctions in this epithelial cell layer (39). The increased rate of zona hatching by EGF could be due to increased plasminogen activator activity of the trophectoderm cells by this growth factor (40).

The present results indicate that two-cell embryos cultured singly become quite sensitive to environmental conditions at the eight-cell/morula stage. This could explain why most of the freshly recovered eight-cell embryos developed to blastocysts when cultured singly even in the absence of any growth factors as opposed to two-cell embryos cultured similarly. It is possible that embryos developed *in vivo* up to the eight-cell stage were already exposed to necessary factors to develop into blastocysts *in vitro*. However, proliferative and zona hatching responses still required the support of growth factors. A decline in cleavage rate from about eight-cell stage *in vitro* compared with that *in vivo* (4) further suggests that the eight-cell/morula stage is critical in the embryo's life.

The model established in this study allows examination of the roles of various growth factors and their interactions on preimplantation embryo development in various species. The study provides important information to our understanding of normal preimplantation embryo development and could be valuable for *in vitro* fertilization, preimplantation diagnosis of genetic diseases, development of serum-free culture medium for embryo manipulation, and cloning of embryos *in vitro*.

We thank Dr. Glen K. Andrews for his advice and suggestion during the course of this study. This work was supported in part by a grant from the National Institutes of Health (HD 12304) and by the Wesley Foundation (Wichita, KS).

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