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Development of Mouse Embryos after Immunoneutralization of Mitogenic Growth Factors Mimics that of Cloned Embryos

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The extent to which mitogenic growth factors influence embryo development is not well characterized. We sought to determine the effect of epidermal growth factor (EGF) and transforming growth factor α (TGFα) on naturally fertilized (in vivo-derived) and in vitro-fertilized mouse embryos, compared with that on cloned (intracytoplasmic nuclear injection-derived) mouse embryos, in which EGF and TGFα expression is markedly reduced. Immunoneutralization of EGF, TGFα, and EGF receptor by using specific antibodies significantly reduced the blastocyst development rate (in vivo-derived: 66%, 63%, and 63%, respectively; in vitro-fertilized: 57%, 55%, and 56%, respectively), increased the number of apoptotic nuclei (in vivo-derived: 9%, 10%, and 9%, respectively; in vitro-fertilized: 13%, 13%, and 13%, respectively), decreased the total number of cells (in vivo-derived: 87%, 85%, and 86%, respectively; in vitro-fertilized: 86%, 85%, and 86%, respectively), and increased the inner cell mass:trophectoderm ratios (in vivo-derived: 1:2.70 ± 0.05, 1:2.73 ± 0.04, 1:2.71 ± 0.06, respectively; in vitro-fertilized: 1:2.94 ± 0.02, 1:2.96 ± 0.02, 1:2.95 ± 0.02, respectively). In most cases, combined treatment with neutralizing antibodies to both EGF and TGFα accentuated changes in these parameters. Further, the effect of combined immunoneutralization on these parameters in fertilized embryos was no different from those in cloned embryos. Therefore, normal expression of mitogenic growth factors is crucial for successful development of mouse embryos before implantation. Inhibiting the action of mitogenic growth factors causes fertilized embryos to exhibit developmental characteristics similar to those of cloned embryos, which may partially explain the poor developmental potential of cloned mammalian embryos.

Abbreviations: BSA, bovine serum albumin; dpc, days postcoitus; EGF, epidermal growth factor; EGFR, EGF receptor; hCG, human chorionic gonadotropin; HTF, human tubal fluid; ICM, inner cell mass; ICNI, intracytoplasmic nuclear injection; IVF, in vitro fertilization; KLH, keyhole limpet hemocyanin; PBS, phosphate buffered saline; PVP, polyvinyl pyrrolidone; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; TE, trophectoderm; TGFα, transforming growth factor; TUNEL assay, TdT-mediated dUTP nick end-labeling assay

Formation of a developmentally competent preimplantation mammalian embryo involves the transition from a unicellular, totipotent zygote to a differentiated, multicellular blastocyst. This progression requires tightly coordinated and appropriately timed regulation of cell division, differentiation, and programmed cell death to ensure the divergence of 2 cell lineages: the pluripotent cells of the inner cell mass (ICM) that give rise to the embryo proper and the surrounding trophectoderm (TE) cells that form the extraembryonic membrane (embryonic placenta). Regulation of this developmental process by soluble factors in embryos is not completely understood.

In addition to a diverse array of cellular responses, mitogenic growth factors initiate an intracellular signal transduction cascade that induces activation of mitogen-activated protein kinase and other signal-regulated kinases; activation of this cascade leads to stimulation of gene expression.[11] Similar to the effect on somatic cells, the effect of mitogenic growth factors on growth and differentiation of cells of the developing embryo has been well articulated.[2] Mitogenic growth factors have profound influence on embryo development,[8,14,23] growth,[23] and differentiation.[1,43] Further, expression of mitogenic growth factors in the maternal reproductive tract appears to be temporally coordinated so as to optimize embryo cleavage potential, implantation, and organogenesis.[26,32]

However, the extent to which expression of mitogenic growth factors is essential for normal development of preimplantation embryos is not known. Previously, we found that expression levels of epidermal growth factor (EGF), transforming growth factor α (TGFα), and their cognate receptor, epidermal growth factor receptor (EGFR), were reduced significantly in preimplantation cloned mouse embryos.[6] Because early development and survival of cloned mouse embryos are notoriously poor, it is reasonable to consider the possibility that reduced expression of mitogenic growth factors may impair normal preimplantation embryo development. Therefore, we investigated the extent to which these growth factors play a role in the development of cloned mouse embryos. We hypothesized that expression of EGF, TGFα, and EGFR is required for normal preimplantation development of naturally fertilized (in vivo-derived) and in vitro-fertilized (IVF) embryos. To test this hypothesis, we measured the effect of immunoneutralizing antibodies to EGF, TGFα, and EGFR on the rate of development of 1-cell-stage embryos into blastocysts and on the number of apoptotic cells, total number of ICM and TE cells, and ICM:TE ratios in fertilized mouse embryos and then

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compared our findings with those from cloned mouse embryos prior to implantation.

Materials and Methods

Experimental design. We allocated 1-cell-stage in vivo-derived (embryo group 1) and in vitro-fertilized (embryo group 2) embryos into 4 treatment groups each according to supplementation of in vitro culture media with neutralizing antibodies to either EGF, TGFα, EGFR, or to both EGF and to TGFα. In addition, 2 groups of each type of embryo were exposed to either no antibodies (negative control) or neutralizing antibodies to keyhole limpet hemocyanin (KLH; positive control).[27] For comparison, cloned embryos (embryo group 3) derived by intracytoplasmic nuclear injection (ICNI) were allocated into positive (anti-KLH) and negative (no antibody) control groups for analysis. B6D2F1 (C57BL/6J × DBA/2J) mice obtained from The Jackson Laboratory (Bar Harbor, ME) were used as sources of germplasm and somatic cells for this study. Mice were kept in a pathogen-free barrier facility according to standard colony management procedures (14:10 light:dark cycle; 1 female per cage; food and water ad libitum). All experiments were conducted with approval of the Institutional Animal Care and Use Committee of University of California, Davis, and in accordance with National Institutes of Health guidelines for the care and use of animals.

Neutralizing antibodies. Monoclonal antibodies, anti-EGF (20 μg/ml; Ab-2, catalog number GF07L), anti-TGFα (10 μg/ml; Ab-3, catalog number GF15L), and anti-EGFR (phosphor-specific; Tyr^{1173}, 10 μg/ml, catalog number 324864), were obtained from EMD Biosciences (Calbiochem, San Diego, CA). Antibodies were prepared specifically for immunoneutralization studies and showed no cross-reactivity. A control antibody, anti-KLH (10 to 20 μg/ml; antibody 30) was a generous gift from Gordon Ohning (CURE/Digestive Diseases Research Center, University of California, Los Angeles). For experiments, immunoneutralizing antibodies were added to culture media (KSOMaa, EmbryoMax, Specialty Media, Phillipsburg, NJ).

Embryo groups. ***Embryo group 1: in vivo-derived embryos.*** B6D2F1 male (8 wk) and female (6 to 8 wk) mice were placed together for mating in the late afternoon of the 1st day. Female mice were examined the following morning for the presence of a vaginal plug indicating coitus (0.5 d postcoitus [dpc]). On the same morning, female mice with vaginal plugs (0.5 d dpc) were separated from the male and were euthanized by CO_2 asphyxiation and cervical dislocation, and the abdomen was exposed to enable dissection of the oviduct and harvest of zygotes (1-cell-stage embryos) from the ampulla. Embryos were collected and placed into 500 μl M2 medium prewarmed to 37 °C, by using standard methodology.[17] Embryos were washed 3 times in the same medium and allocated randomly into the 4 treatment and 2 control groups of approximately 20 to 25 1-cell embryos each and transferred to culture dishes containing fresh, equilibrated KSOMaa medium supplemented with either neutralizing antibodies (20 μg/ml anti-EGF, 10 μg/ml anti-TGFα, 10 μg/ml anti-EGFR, or both 20 μg/ml anti-EGF and 10 μg/ml TGFα), or with complementary doses of the positive control antibody (anti-KLH 20 μg/ml and 10 μg/ml) or no antibodies as a negative control as described previously. Embryos were incubated under embryo-tested mineral oil (Sigma, St Louis, MO) for the next 3 d (until 3.5 dpc) in 5% CO_2 and 95% O_2 at 37 °C.

Embryo group 2: in vitro-fertilized embryos. The IVF methods described in the following text are summarized from our previous publication.[6]

Oocytes. B6D2 F1 female mice (6 to 8 wk) were superovulated by intraperitoneal injection of 5 to 7 IU pregnant mare serum gonadotropin (Sigma) followed 46 h later by intraperitoneal injection of 5 to 7 IU human chorionic gonadotropin (hCG; Sigma). At 13 h after hCG injection, female mice were euthanized by CO_2 asphyxiation and thoracotomy, and the abdomen was exposed to enable dissection of the oviduct and harvest of oocyte–cumulus complexes from the ampulla. The harvested complexes were collected into 1 ml M2 medium warmed to 37 °C, by using standard methodology.[17] Oocyte–cumulus complexes were washed 3 times in the same medium and transferred into 500 μl human tubal fluid (HTF; EmbryoMax, Specialty Media) medium with 4 mg/ml bovine serum albumin (BSA, Sigma) preincubated in 5% CO_2 and 95% O_2 at 37 °C under embryo-tested mineral oil (Sigma).

Sperm collection. Just before female mice were euthanized, B6D2F1 male mice (8 wk) were euthanized by CO_2 asphyxiation and thoracotomy, and fresh sperm was collected from both cauda epididymides by using standard methodology.[34] Sperm was immediately dispersed into 400 μl HTF medium supplemented with 4 mg/ml BSA, preincubated for 12 h in 5% CO_2 and 95% O_2 at 37 °C, and capacitated in the same medium for 1.5 h in 5% CO_2 and 95% O_2 at 37 °C under embryo-tested mineral oil (Sigma).

IVF. Approximately 20 to 25 metaphase-II oocytes with cumulus cells were transferred from M2 medium into 50 μl HTF medium supplemented with 4 mg/ml BSA (EmbryoMax, Specialty Media). As described previously,[16] 10 to 15 μl sperm suspension with a concentration of 1.8×10^6 cells/ml was coincubated with previously harvested oocytes at 37 °C in 5% CO_2 and 95% O_2 for 4 h. Fertilized oocytes were washed 3 times in 50 μl with HEPES–KSOM and mKSOMaa (EmbryoMax, Specialty Media) to remove sperm. After the fertilization rate was assessed (number of oocytes with pronulcei/total number of oocytes × 100%), 1-cell stage embryos were allocated randomly into the 4 treatment and 2 control groups of approximately 20 to 25 1-cell-stage embryos each and transferred to culture dishes containing fresh equilibrated KSOMaa medium supplemented with either neutralizing antibodies, positive control antibody, or no antibody (negative control) as described previously. Embryos were incubated under embryo-tested mineral oil (Sigma) until 3.5 to 4 dpc in 5% CO_2 and 95% O_2 at 37 °C.

Embryo group 3: ICNI-derived embryos. The ICNI methods described are a summary of those presented in our previous publication.[6]

Oocyte-cumulus complexes. Oocyte–cumulus complexes from the same strain (B6D2F1) of superovulated female mice were collected and transferred from M2 medium (see superovulation and collection procedure described earlier) into HEPES-CZB medium (EmbryoMax, Specialty Media). Cumulus cells were dissociated from oocytes by careful incubation of oocyte–cumulus complexes with 100 μl of HEPES-CZB medium containing 300 U/ml bovine testis hyaluronidase (Sigma) for 3 to 5 min on a microscope stage prewarmed to 37 °C. Afterward, dissociated oocytes were removed from the hyaluronidase solution and washed 3 times in mKSOMaa medium and incubated in this medium at 37 °C under 5% CO_2 in air before enucleation, whereas cumulus cells were kept in hyaluronidase solution at 4 °C until isolation of nuclei. ICNI was performed within 3 h of oocyte collection as described previously.[6]

Enucleation. Enucleation was performed as previously described[39] with minor modification. Briefly, 10 metaphase-II oocytes were transferred from mKSOMaa into a drop (approxi-

mately 50 µl) of HEPES-CZB containing 5 µg/ml cytochalasin B (Sigma) under embryo-tested mineral oil in a plastic culture dish. First, the chromosome–spindle complex of an oocyte was identified, a Piezo micromanipulator (PMM controller, PMAS-CT150, Prime Tech, Ibaraki, Japan) then was applied to penetrate the zona pellucida, and the chromosome–spindle complex was aspirated into the enucleation pipette (inner diameter, 10 to 15 µm; B100-75-10, Sutter Instrument Company, Novato, CA) with care to minimize removal of cytoplasm. After enucleation, cytoplasts (enucleated oocytes) were washed and incubated in cytochalasin B-free CZB for no more than 2 h before ICNI.

Donor nuclei. Cumulus cells in approximately 100 µl hyaluronidase solution were transferred into a 1.5-ml microtube containing 1 µl HEPES-CZB medium and centrifuged at 300 × *g* for 2 min, and the cell pellet was resuspended in 30 µl HEPES-CZB medium. The cumulus cell suspension (1 part) was thoroughly mixed with 9 parts 10% PVP in HEPES-CZB containing 0.01% polyvinyl pyrrolidone (PVP) without BSA. Cumulus cells (approximate diameter, 10 to 12 µm) were aspirated gently into and out of an injection pipette (approximate inner diameter, 6 µm) until nuclei were largely devoid of visible cytoplasmic contents. Isolated nuclei were transferred into a new 10% PVP drop and washed; 5 to 8 karyoplasts (extracted nuclei) then were aspirated into the injection pipette for ICNI.

ICNI. The tip of the injection pipette containing the cumulus cell cytoplast was moved into contact with the zona pellucida. Penetration of the zona pellucida was facilitated by the application of a few piezoelectric pulses. A plug of the zona was aspirated into the injection pipette and subsequently expelled from the injection pipette into the perivitelline space. The injection pipette was advanced into the oolema until the tip reached the opposite side of the ooplasm, at which time the oolema was penetrated by applying a weak piezoelectric pulse. One cumulus cell karyoplast was injected into the cytoplasm with a small amount of medium, and the pipette was gently withdrawn. After injection, reconstructed oocytes were washed and incubated in CZB or mKSOMaa medium for 1 to 3 h before activation. All micromanipulations were performed at room temperature.

Activation. Activation was induced by incubation of reconstructed oocytes in 50 µl Ca^{2+}-free CZB containing both 10 mM Sr^{2+} (Sigma, St Louis, MO) and 5 µg/ml cytochalasin B for 6 h at 37 °C under 5% CO_2 in air. After activation, reconstructed oocytes were allocated into 2 treatment subgroups of approximately 20 to 25 1-cell-stage embryos each and transferred to culture medium supplemented with either positive-control antibody or no antibody (negative control) as described previously.

Embryo culture. Every 24 h until blastocyst stage, 1-cell-stage embryos were transferred to fresh drops of 50 µl KSOMaa for culture in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37 °C under a layer of embryo-tested mineral oil. Development of embryos to the 2-cell, 4-cell, morula, and blastocyst stages in each treatment group was recorded. Embryos were assessed morphologically for differentiation to ICM and TE cells and for blastocoel formation to ensure that nearly all reached the blastocyst stage approximately 96 to 110 h post-hCG. The rate of development of 1-cell stage-embryos to blastocyst stage (100% × number of blastocysts/total number of 1-cell embryos) was calculated immediately prior to terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay, differential staining, and cell counting.

Assays. ***TUNEL Assay.*** To determine the amount of apoptosis affecting in vivo-derived, in vitro-fertilized, and ICNI-derived cloned embryos, blastocysts with intact zona pellucida were selected randomly from each group and fixed, permeabilized, and stained as described previously.[3] Briefly, blastocysts were transferred into 50 µl of a 1-mg/ml solution of PVP in phosphate buffered saline (PBS; PVP-PBS), and fixed in 4% paraformaldehyde in PBS for 1 h at room temperature or overnight at 4 °C. After fixation, embryos were washed 3 times in PBS-PVP, followed by permeabilization in 0.5% Triton X100 in PBS for 1 h at room temperature. Embryos were incubated in and labeled using fluorescein-conjugated dUTP and TdT (TUNEL reagents, Boehringer Mannheim, Indianapolis, IN) for 1 h at 37 °C in dark, and then washed twice in 0.5% Triton X100-PBS and once in PBS-PVP. Embryos were counterstained by incubating in 50 µg/ml propidium iodide (PI) and RNAse A for 1 h at room temperature in dark. Embryos were washed 2 times in 0.5% Triton X100-PBS solution and once in PBS-PVP, mounted on a slide in 15 µl antifade solution (50% glycerol in PBS), and covered with a coverslip under gentle pressure. Finally, embryos were observed under fluorescence microscope by using a blue filter (excitation wavelength, 450 to 490 nm) for TUNEL-labeled nuclei and a green filter (excitation wavelength, 512 to 560 nm) for PI-stained cells. To determine DNA fragmentation, fluorescein isothiocyanate-labeled green fluorescent deposits (fluorescein–dUTP–TdT) in the nuclei were visualized as bright-yellow when counterstained with PI and were counted as apoptotic cells (fragmented nuclei). PI-labeled (red) nuclei were counted as intact cells (nonfragmented nuclei). The apoptotic index was calculated as: number of dead (yellow) cells/total number of cells (red and yellow). Blastocysts designated for positive controls were preincubated in DNase (50 U/ml) before labeling in TUNEL reagents, and negative controls were incubated in fluorescein–dUTP without TdT enzyme.

Number of cells and differential labeling. To determine to what extents the numbers of cells and ICM:TE ratio were affected by treatment with neutralizing antibodies, ICM and TE cells were differentially labeled using PI and bisbenzimide (Hoechst 33258) by using a modification of a previously published protocol.[38] Briefly, blastocysts with intact zona pellucida were incubated in 500 µl HEPES-buffered HTF (Specialty Media) containing 1% Triton X100 and 100 µg/ml PI for 10 s, during which time changes in the color and size of TE cells could be seen microscopically. Blastocysts were transferred immediately into 500 µl 100% ice-cold ethanol containing 25 µg/ml bisbenzimide and incubated overnight at 4 °C. Fixed and stained blastocysts were mounted on a glass slide in 5 µl glycerol and gently flattened with a coverslip, and fluorescence was visualized under a fluorescence microscope. To analyze the total number of cells, cells were visualized using a filter for DAPI (330 to 380 nm), whereas differentially labeled ICM and TE cells were visualized using different filters (460 nm for blue and red fluorescence, and 560 nm for red only).

Statistics. All data were analyzed using the SPSS 11.0 statistical program (Stat/Math Center, UITS Research and Academic Computing Division, UCD). Univariate analysis of variance and Tukey honest significant difference (HSD) multiple comparison procedures for marginal means (post hoc tests), F tests, and homogeneous subsets were performed for all experimental and treatment groups. A P value of less than 0.05 was considered statistically significant for all data, which are presented as mean ± standard error.

Table 1. Rate and percentage (mean ± SE) of development of 1-cell stage to blastocyst stage of in vivo-derived, in vitro-derived, and ICNI-derived (cloned) embryos after treatment with neutralizing antibodies to EGF, TGFα, and EGF-R; combined treatment with EGF and TGFα; and negative (no antibody) and positive (anti-KLH) control treatments

Embryo group	Control treatments		Neutralizing antibody treatments			
	Negative	Positive	EGF	TGFα	EGF-R	EGF + TGFα
In vitro-fertilized	36.2 ± 0.50[a] (86%)	35.3 ± 0.49[a] (86%)	27.3 ± 0.83[b*] (66%)	26.5 ± 0.64[b*] (63%)	26.3 ± 0.70[b*] (63%)	12.6 ± 0.61[c†] (40%)
In vitro-derived	30.3 ± 0.53[d] (82%)	30.4 ± 1.01[d] (83%)	22.6 ± 0.25[e†] (57%)	21.5 ± 0.22[e†] (55%)	21.2 ± 0.58[e†] (56%)	11.8 ± 0.37[c†] (36%)
ICNI-derived (cloned)	4.5 ± 0.30[f] (35%)	4.9 ± 0.34[f] (35%)	Not determined	Not determined	Not determined	Not determined

Values with different alphanumeric superscripts are significantly ($P < 0.05$) different.
Symbols indicate statistically significant (*, $P < 0.05$; †, $P < 0.01$) differences from control treatments within an embryo group.

Results

Rate of development and embryo morphology. Compared with those after control antibody treatments, the numbers of blastocysts (mean ± standard error) that developed from in vivo-derived 1-cell-stage embryos were significantly ($P < 0.05$) reduced after treatment with neutralizing antibodies to EGF (27.3 ± 0.8 blastocysts), TGFα (26.5 ± 0.6 blastocysts), and EGFR (26.3 ± 0.7 blastocysts; Table 1). Similar relative results were observed after treatment of in vitro-fertilized embryos with neutralizing antibodies to EGF (22.6 ± 0.3 blastocysts), TGFα (21.5 ± 0.2 blastocysts), and EGFR (21.2 ± 0.6 blastocysts). Compared with that of in vivo-derived embryos, development of in vitro-fertilized embryos was poorer after immunoneutralization. Further, the numbers of blastocysts that developed after combined treatment with neutralizing antibodies to both EGF and TGFα of in vivo-derived embryos (12.6 ± 0.6 blastocysts) and in vitro-fertilized embryos (11.8 ± 0.4) were significantly ($P < 0.01$) fewer than after treatment with either control or individual neutralizing antibodies. After combined antibody treatment, the percentage of blastocysts that developed from in vivo-derived (40%) and in vitro-fertilized (36%) embryos was no different from the percentage of blastocysts that developed from ICNI-derived (cloned) embryos (35%). Further, after immunoneutralization, embryos were characterized morphologically by shrinkage and fragmentation of nuclear chromatin. These changes were pronounced among in vitro-fertilized embryos after antibody treatment and in cloned embryos (Figure 1 A, D, and G).

Apoptosis. Apoptosis, or programmed cell death, is an important parameter by which to assess the developmental competence of preimplantation mammalian embryos. Apoptotic cells were detected in all groups of embryos after treatment with neutralizing antibodies (Table 2 and Figure 1 B and E). Compared with that after control antibody treatment, the numbers of cells (mean ± SE) with apoptotic nuclei among in vivo-derived embryos were significantly ($P < 0.05$) greater after treatment with neutralizing antibodies to EGF (7.3 ± 0.4 cells), TGFα (7.7 ± 0.5 cells), and EGFR (7.5 ± 0.3 cells) and combined treatment with antibodies to both EGF and TGFα (10.3 ± 0.3 cells). Similar relative and statistically significant ($P < 0.01$) results were observed after treatment of in vitro-fertilized embryos with neutralizing antibodies to EGF (9.4 ± 0.3 cells), TGFα (9.6 ± 0.4 cells), and EGFR (9.4 ± 0.2) and combined treatment with antibodies to both EGF and TGFα (10.8 ± 0.5 cells). In both groups of embryos, after treatment with neutralizing antibodies, 92% to 94% of apoptotic cells were in the ICM (Figure 1 B and E). Further, after combined antibody treatment, the percentage of apoptotic nuclei in in vivo-derived (13%) and in vitro-fertilized (15%) embryos was no different from that in ICNI-derived cloned embryos (15%; Table 2 and Figure 1 E and H). Linear regression analysis revealed that the number of apoptotic cells was negatively correlated ($R^2 = 0.77$) with the total number of cells (Figure 2).

Number of cells and differential labeling. As the total number of embryonic cells decreases, the developmental potential of preimplantation mammalian embryos decreases. Compared with that after control antibody treatments, the total number of cells (mean ± SE) in in vivo-derived embryos was significantly ($P < 0.05$) less after treatment with neutralizing antibodies to EGF (68.8 ± 0.9 cells), TGFα (67.8 ± 1.6 cells), and EGFR (68.0 ± 2.2 cells) and combined treatment with antibodies to both EGF and TGFα (59.4 ± 0.7 cells; Table 3 and Figure 1 A, D, and G). Similar relative results were observed after treatment of in vitro-fertilized embryos with neutralizing antibodies to EGF (63.8 ± 0.7 cells), TGFα (63.0 ± 1.2 cells), and EGFR (63.6 ± 1.3 cells) and combined treatment with antibodies to both EGF and TGFα (57.4 ± 0.5). Cell size was decreased after immunoneutralization treatment (Figure 1 D). Further, after combined antibody treatment, the percentage of total number of cells in in vivo-derived (77%) and in vitro-fertilized (77%) embryos was no different from that in ICNI-derived embryos (78%; Table 3, Figure 1 G).

ICM:TE ratio. The ratio of ICM:TE cells is an indication of differences in the pattern of the cell division and rate of cell death between the 2 cell lineages in early preimplantation embryos (blastocysts). Compared with that after control antibody treatments, the ICM:TE ratios (mean ± SE) of in vivo-derived embryos did not differ ($P = 0.097$) after treatment with individual neutralizing antibodies but were significantly ($P < 0.01$) different after combined antibody treatment (1:3.16 ± 0.03; Table 4, Figure 1 F). In contrast, significantly ($P < 0.05$) different relative results were observed after treatment of in vitro-fertilized embryos with neutralizing antibodies to EGF (1:2.94 ± 0.02), TGFα (1:2.96 ± 0.02), and EGFR (1:2.95 ± 0.02) and combined treatment with antibodies to both EGF and TGFα (1:3.24 ± 0.02). Further, after combined antibody treatment, the mean ICM:TE ratios of fertilized embryos were not significantly different from those of cloned embryos (1:3.21 ± 0.03; Table 4, Figure 1 C, F, and I).

Discussion

In the present study we determined that the development of mouse embryos derived by either natural mating or IVF was negatively affected by immunoneutralization of mitogenic growth factors and their receptor. Treatment with immunoneutralizing antibodies to EGF, TGFα, and EGFR decreased the rate of development of 1-cell-stage embryos to blastocysts, reduced the total number of differentiated cells in the ICM and TE, decreased cell size, reduced the ICM:TE ratio, and increased the fragmentation of nuclear DNA (that is, apoptosis). These differences were accentuated, in most cases, when embryos were treated simultaneously with neutralizing antibodies to both EGF and TGFα. In vitro-fer-

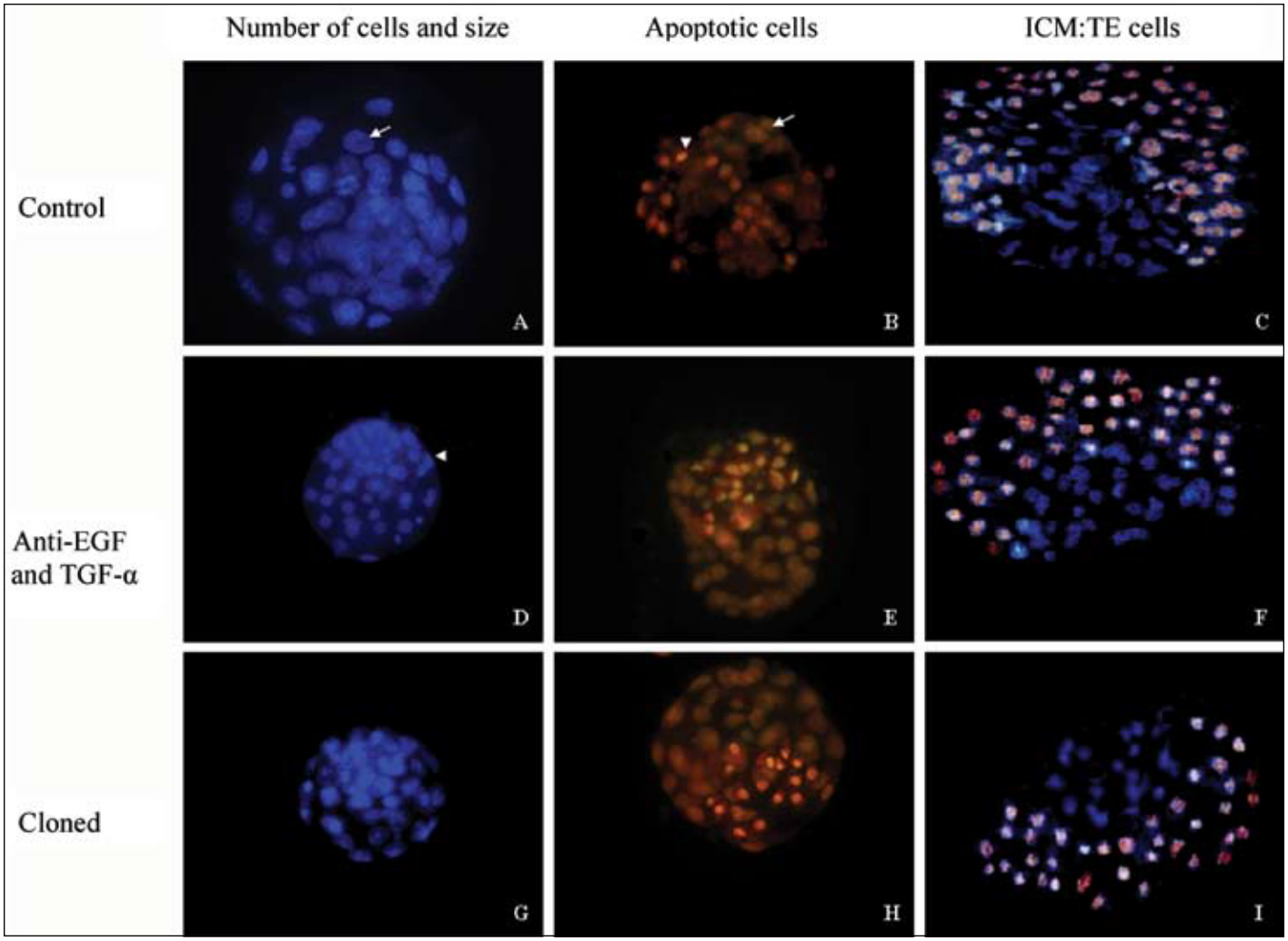

Figure 1. Fluorescent labeling of cells after control and neutralizing antibody treatments of fertilized embryos compared with ICNI-derived (cloned) embryos ([A, D, and G]; arrow points to normal-appearing cells, and arrowhead points to markedly shrunken cells), apoptotic cells ([B, E, and H]; arrow points to normal appearing cells [red to red-green], and arrowhead points to apoptotic cells [yellow]), and differentiation (C, F, and I) of ICM (blue) and TE (red to pink). Magnification, ×630; resolution, 1300 × 1030 pixels at 150 pixels per in.

Table 2. Number and percentage (mean ± SE) of apoptotic cells in in vivo-derived, in vitro-derived, and ICNI-derived (cloned) embryos after treatment with neutralizing antibodies to EGF, TGFα, and EGF-R; combined treatment with EGF and TGFα; and negative (no antibody) and positive (anti-KLH) control treatments

Embryo group	Control treatments		Neutralizing antibody treatments			
	Negative	Positive	EGF	TGFα	EGF-R	EGF+ TGFα
In vitro-fertilized	3.4 ± 0.19^{a} (4%)	3.7 ± 0.26^{a} (5%)	$7.3 \pm 0.37^{b*}$ (9%)	$7.7 \pm 0.54^{b*}$ (10%)	$7.5 \pm 0.33^{b*}$ (9%)	$10.3 \pm 0.30^{c\dagger}$ (13%)
In vitro-derived	3.8 ± 0.37^{a} (5%)	4.0 ± 0.32^{a} (5%)	$9.4 \pm 0.25^{c\dagger}$ (13%)	$9.6 \pm 0.40^{c\dagger}$ (13%)	$9.4 \pm 0.24^{c\dagger}$ (13%)	$10.8 \pm 0.45^{c\dagger}$ (15%)
ICNI-derived (cloned)	10.5 ± 0.58^{c} (15%)	10.4 ± 0.51^{c} (15%)	Not determined	Not determined	Not determined	Not determined

Values with different alphanumeric superscripts are significantly ($P < 0.05$) different.
Symbols indicate statistically significant (*, $P < 0.05$; †, $P < 0.01$) differences from control treatments within an embryo group.

tilized embryos appeared to be more sensitive than in vivo-derived embryos to immunoneutralizing antibodies. Therefore, the negative effect of neutralizing antibodies to mitgoegnic growth factors on the growth and differentiation characteristics of fertilized embryos indicates a direct relationship between the actions of EGF and TGFα and normal development of mouse embryos.

In comparison, the changes observed in ICNI-derived cloned mouse embryos were very similar to those in both in vivo-derived and in vitro-fertilized embryos after immunoneutralization of mitogenic growth factors. Cloned embryos showed reductions in the rate of blastocyst development, numbers and ratio of ICM and TE cells, and total number of differentiated cells and increases in the frequency of apoptotic nuclei identical to those after combined treatment of fertilized embryos with neutralizing antibodies to EGF and TGFα. Except for the mean number of apoptotic cells in in vitro-fertilized embryos, the similarities between fertilized and cloned embryos were made only after combined antibody treatment. These results are consistent with findings from our

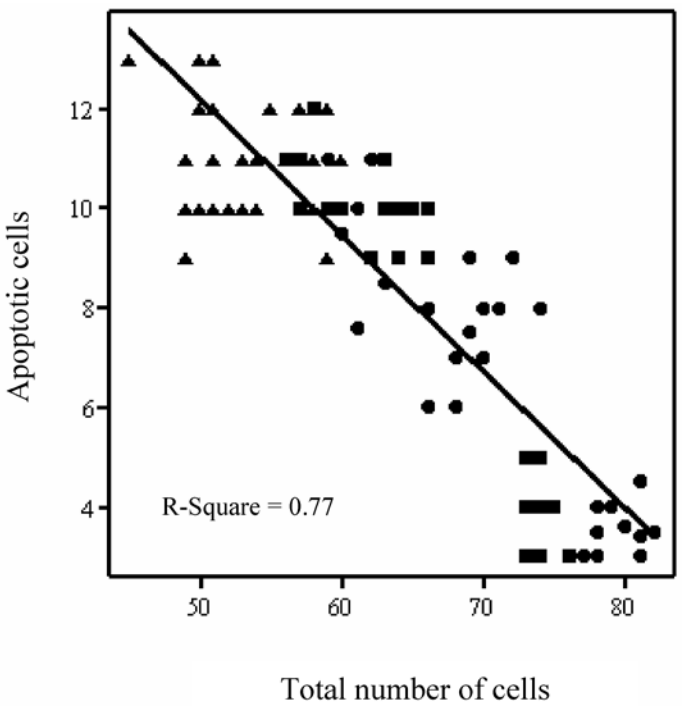

Figure 2. Linear regression plot demonstrating negative correlation (R^2 = 0.77) between apoptotic cells and total number of cells in in vivo-derived (●), in vitro-fertilized (■), and ICNI-derived (cloned; ▲) embryos.

previous study in which ICNI-derived cloned embryos exhibited reduced levels of expression of EGF, TGFα, and EGFR.[6] Although the etiology remains unknown, these results strongly suggest that altered expression of EGF, TGFα, and EGFR is related to poor development of cloned mouse embryos.

Our findings are consistent with results that indicate a lower rate of development and a greater incidence of programmed cell death (that is, apoptosis) in IVF and cloned embryos than in vivo-derived embryos.[3,15] To a variable extent, these earlier observations were dependent on culture conditions,[10,13,19,20] which likely affect gene expression and nuclear reprogramming.[7,33,36] Our experimental design controlled for this variable by applying identical culture conditions to all 3 embryo groups. In this way, we could compare and contrast results between fertilized and cloned embryos in order to specifically address the role of mitogenic growth factors in preimplantation embryo development.

Although DNA fragmentation and apoptosis are normal features of mammalian embryo development,[3,30] we found that the extent of apoptosis increased in embryos after treatment with neutralizing antibodies and became particularly prominent in cells of the ICM. These findings are not unexpected and are consistent with previous reports showing that cell death in blastocysts occurs primarily in cells of the ICM and only occasionally in cells of the TE.[3,4,13] Recent results also have revealed a negative correlation between the frequency of DNA-fragmented nuclei and the total number of cells in in vitro-fertilized and cloned blastocysts,[10,24] suggesting that differences in cell death between the ICM and TE are likely due to differences in the patterns of cell proliferation and rate of cell death after formation of these 2 cell lineages.[37]

The use of immunoneutralizing antibodies to study the role of mitogenic growth factors on embryo development has been validated previously.[5,18] Antibodies to EGFR that specifically interact with and block an epitope corresponding to the major autophosphorylation site of the EGFR have been shown to inhibit activity of the intracellular cascade induced by mitogen-activated protein kinase and receptor tyrosine kinase, thus negatively affecting preimplantation embryo development.[22,25,27,42] The effect of blocking this critical cytoplasmic signaling mechanism is to decrease amino acid uptake, protein synthesis, Na^+/K^+-ATPase activity, and Na^+–K^+–Cl^- transport.[40,41] These changes are reflected in a reduction in cell size, which is consistent with our findings. Further, the absence of any effect of anti-KLH antibodies (positive control) compared with no antibody treatment (negative control) on embryo development eliminates nonspecific and toxic effects of antibodies on embryo development. These factors all provide a high degree of assurance that the changes in developmental characteristics that we measured in fertilized embryos were affected specifically by immunoneutralization of mitogenic growth factors.

Table 3. Total number and percentage (mean ± SE) of cells in in vivo-derived, in vitro-derived, and ICNI-derived (cloned) embryos after treatment with neutralizing antibodies to EGF, TGFα, and EGF-R; combined treatment with EGF and TGFα; and negative (no antibody) and positive (anti-KLH) control treatments

Embryo group	Control treatments		Neutralizing antibody treatments			
	Negative	Positive	EGF	TGFα	EGF-R	EGF+ TGFα
In vitro-fertilized	79.4 ± 0.68^{a} (100%)	79.6 ± 0.93^{a} (100%)	68.8 ± 0.86^{b*} (87%)	67.8 ± 1.6^{b*} (85%)	68.0 ± 2.2^{b*} (86%)	59.4 ± 0.68^{c†} (77%)
In vitro-derived	73.8 ± 0.37^{a} (93%)	74.0 ± 0.55^{a} (93%)	63.8 ± 0.73^{e*} (86%)	63.0 ± 1.2^{e*} (85%)	63.6 ± 1.3^{e*} (86%)	57.4 ± 0.51^{c†} (77%)
ICNI-derived (cloned)	58.0 ± 0.71^{c} (78%)	57.6 ± 1.0^{c} (78%)	Not determined	Not determined	Not determined	Not determined

Values with different alphanumeric superscripts are significantly ($P < 0.05$) different.
Symbols indicate statistically significant (*, $P < 0.05$; †, $P < 0.01$) differences from control treatments within an embryo group.

Table 4. ICM:TE ratios (mean ± SE) in in vivo-derived, in vitro-derived, and ICNI-derived (cloned) embryos after treatment with neutralizing antibodies to EGF, TGFα, and EGF-R; combined treatment with EGF and TGFα; and negative (no antibody) and positive (anti-KLH) control treatments

Embryo group	Control treatments		Neutralizing antibody treatments			
	Negative	Positive	EGF	TGFα	EGF-R	EGF + TGFα
In vitro-fertilized	1:2.64 ± 0.02^{a}	1:2.65 ± 0.02^{a}	1:2.70 ± 0.05^{a}	1:2.73 ± 0.04^{a}	1:2.71 ± 0.06^{a}	1:3.16 ± 0.03^{b†}
In vitro-derived	1:2.72 ± 0.03^{a}	1:2.73 ± 0.03^{a}	1:2.94 ± 0.02^{c*}	1:2.96 ± 0.02^{c*}	1:2.95 ± 0.02^{c*}	1:3.24 ± 0.02^{b†}
ICNI-derived (cloned)	1:3.20 ± 0.03^{b}	1:3.21 ± 0.03^{b}	Not determined	Not determined	Not determined	Not determined

Values with different alphanumeric superscripts are significantly ($P < 0.05$) different.
Symbols indicate statistically significant (*, $P < 0.05$; †, $P < 0.01$) differences from control treatments within an embryo group.

Mitogenic growth factors are thought to play key regulatory roles in preimplantation embryo development by promoting nuclear and cytoplasmic maturation,[8,31] increasing fecundity and rates of fertilization,[12,28,35] optimizing cellular cleavage, and synchronizing embryonic and maternal maturation.[9,14,21] Our results, in the context of our earlier work,[6] strongly support the contention that mammalian embryos are particularly sensitive to the presence of mitogenic growth factors and that adequate levels and appropriately timed expression of these factors are crucial for successful preimplantation embryo development. This necessary role in development indicates that EGF and TGFα expression and in turn EGFR activation are essential for ensuring appropriate cell proliferation and differentiation and thus promote survival and normal preimplantation development.[9,29] Therefore, our results indicate that the rate of embryo development, total number of differentiated cells, rate of programmed cell death, and ICM:TE ratio are useful parameters for assessing the developmental competence of preimplantation cloned mouse embryos. For example, the onset of apoptosis is related to the start of activation of the embryonic genome, which takes place at the 2-cell stage.[3,19]

In summary, immunoneutralization of endogenous EGF, TGFα, and EGFR disrupts preimplantation development of in vivo-derived and in vitro-fertilized mouse embryos. As reported, the ultimate effect is premature and extensive apoptosis leading to a low rate of embryo development, similar to that seen in cloned embryos. These observations complement findings from our previous study[6] in which cloned embryos revealed severely impaired expression of mitogenic growth factor genes, thus indicating a critical role in preimplantation embryo development. Whether mitogenic growth factors are necessary and sufficient for normal development and survival of mouse embryos awaits future studies to determine whether supplementation with EGF and TGFα can improve the developmental potential of cloned mouse embryos.

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