

# Decreased Growth Factor Expression through RNA Interference Inhibits Development of Mouse Preimplantation Embryos

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Mitogenic growth factors play an important role in cellular development and differentiation. The purpose of this study was to assess the extent to which epidermal growth factor (EGF) and transforming growth factor  $\alpha$  (TGF $\alpha$ ) and their cognate receptor (EGFR) are crucial for normal preimplantation embryo development. We used RNA interference to decrease expression of growth factors in preimplantation mouse embryos. We microinjected 1-cell mouse embryos individually with short interfering RNA (siRNA) specific to *EGF*, *TGF $\alpha$* , and *EGFR* and then analyzed temporal and spatial gene expression patterns at different stages of development before implantation. Transfection with siRNA significantly reduced growth factor expression in 1-cell, 2-cell, morula, early-blastocyst, and late-blastocyst embryos to levels similar to those in untreated 'cloned' embryos derived through intracytoplasmic nuclear injection. In addition, siRNA effectively decreased expression of maternally inherited genes between 24 and 72 h after transfection, with recovery of gene expression during late-blastocyst stage at 96 h after transfection. Furthermore, siRNA significantly decreased blastocyst formation, increased the number of apoptotic cells, and reduced the total number of differentiated cells in blastocysts; these changes were greatest after decreasing *EGFR* and of both *EGF* and *TGF $\alpha$*  simultaneously. These results support our hypothesis that EGF and TGF $\alpha$  are crucial for embryo survival and development. Further, dysregulated expression of growth factors is associated with poor development of cloned mouse embryos.

**Abbreviations:** EGF, epidermal growth factor; EGFR, EGF receptor; BLAST, basic local alignment search tool; FHM, flushing–holding medium; ICM, inner cell mass; ICNI, intracytoplasmic nuclear injection; KSOM, potassium simplex optimized medium; RNAi, RNA interference; siRNA, short interfering RNA; TE, trophoectoderm; TGF $\alpha$ , transforming growth factor  $\alpha$ ; CZB, Chatot Ziomek Bavister.

Mammalian embryonic development is a complex process that involves cell–cell signaling, cell migration, differentiation, apoptosis, and a host of other biological processes. Regulation of embryo development is mediated by specific temporal and spatial expression of numerous growth factors, including epidermal growth factor (EGF) and transforming growth factor  $\alpha$  (TGF $\alpha$ ).<sup>4,6,27</sup> By activating specific intracellular signaling cascades, growth factors regulate cell proliferation and differentiation and prevent apoptosis.<sup>9</sup> Genes encoding these growth factors and their cognate receptor (EGFR) are expressed sequentially by cells of the maternal reproductive tract and embryo, optimizing cell cleavage and differentiation during early embryogenesis.<sup>31,32</sup>

In our previous studies comparing the timing, pattern, and level of expression of EGF, TGF $\alpha$ , and EGFR in mouse embryos,<sup>10</sup> we found that gene expression levels are maternally derived in fertilized oocytes and then decline after fertilization until the 2-cell stage, when embryo-derived gene expression is activated and steadily increases until the blastocyst stage. In contrast, growth factor gene expression in cloned mouse embryos fails to increase significantly above baseline. These results are consistent with our earlier work using growth factor immunoneutralization in natu-

rally derived mouse embryos<sup>11</sup> and growth factor replacement in cloned mouse embryos,<sup>12</sup> which focused on blocking and replacing, respectively, growth factor function.

In contrast, posttranscriptional gene silencing using double-stranded RNA targeted to specific growth factors to induce RNA interference (RNAi) has proven to be a powerful tool to study the function of many endogenous genes in various organisms, including mammals.<sup>16,17,39,40</sup> Therefore, in the present study, we sought to determine whether RNAi technology could be used to determine the extent to which growth factor gene expression contributes to the poor survival and development of cloned embryos. After treating embryos derived through natural mating with gene-specific siRNA, we analyzed and correlated gene expression with objective measures of preimplantation development, and we compared these findings to those in cloned embryos derived by intracytoplasmic nuclear injection (ICNI). These results support our previous findings<sup>10–12</sup> and indicate that uninterrupted expression of mitogenic growth factors are crucial for embryo survival and development and that deficient expression of these genes leads to poor developmental competence in preimplantation cloned embryos.

## Materials and Methods

**Experimental design.** Four siRNA experimental treatment groups (EGF, TGF $\alpha$ , EGFR, and combined EGF and TGF $\alpha$ ), 2 control groups (no siRNA treatment as negative control and non-

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inhibitory siRNA as positive control), and an untreated 'cloned' embryo group were used. The siRNA and noninhibitory siRNA were purchased from Invitrogen (Carlsbad, CA). Embryos for both siRNA and control treatments were derived by natural mating, and cloned embryos were obtained through ICNI. B6D2F1 (C57BL/6J × DBA/2J) male and female mice (age, 6 to 8 wk) were obtained from Jackson Laboratory (Bar Harbor, ME) and were kept in pathogen-free barrier facility according to standard colony management procedures (14:10-h light:dark cycle, 1 mouse per cage; food and water ad libitum). For each treatment and control group, 15 female mice mated to male mice were used to harvest approximately 150 embryos (1-cell stage), which were divided into 5 replicate trials of 30 embryos per treatment. In addition, 150 mature oocytes were harvested from other B6D2F1 female mice for use in cloning by ICNI. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee of University of California, Davis.

**siRNA synthesis.** Specific nucleotide sequences for targeted genes (*EGF*, *TGF $\alpha$* , and *EGFR*) were selected for siRNA constructs from mouse (*Mus musculus*) cDNA sequences and according to published guidelines.<sup>15</sup> siRNA constructs were designed online by using Block-iT RNAi Designer (Invitrogen). Selected sequences were sorted and screened in a basic local alignment search tool BLAST search<sup>1</sup> to verify that the mRNAs specific for genes of interest were targeted, and siRNA duplexes corresponding to the genes of interest were custom-synthesized. The siRNA sequences for *EGF* (locus 13645; GenBank accession number, NM\_010113) were: sense RNA, 5' GCA GAC AUG GAU AUU CAU U 3'; antisense RNA, 5' AAU GAA UAU CCA UGU CUG C 3'; for *TGF $\alpha$*  (locus 21802; accession number, NM\_031199): sense, 5' CCC ACA CUC AGU ACU GCU U 3'; antisense, 5' AAG CAG UAC UGA GUG UGG G 3'; and *EGFR* (locus 13649; accession number, NM\_207655): sense, 5' GGA AAU UAC CUA UGU GCA A 3'; antisense, 5' UUG CAC AUA GGU AAU UUC C 3'. Synthetic oligonucleotides were HPLC-purified, and siRNA were generated by mixing 20  $\mu$ M of each sense and antisense RNA 19mer in annealing buffer as previously described.<sup>15</sup> Embryos from natural mating and either transfected with noninhibitory siRNA or non-transfected were used as controls.

**siRNA microinjection.** Purified siRNAs were microinjected as described previously.<sup>18,20</sup> Briefly, 1-cell stage embryos were collected into warm flushing–holding medium (FHM) from the oviducts of naturally mated B6D2F1 female mice that were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin, followed 48 h later by injection of 5 IU human chorionic gonadotrophin. Mice with vaginal plugs at 0.5 d post coitus were euthanized by CO<sub>2</sub> asphyxiation and cervical dislocation.

Cumulus cells attached to zygotes were removed by brief incubation (2 to 3 min) in FHM containing 300 U/mL bovine testis hyaluronidase at room temperature. Zygotes then were washed and cultured in potassium simplex optimized medium (KSOM, Specialty Media, Phillipsburg, NJ), covered with mineral oil, and placed in 5% CO<sub>2</sub> in humidified air at 37.5 °C before microinjection of siRNA. Microinjection was performed on an inverted microscope (TE300, Nikon Instruments, Melville, NY) with micromanipulators (Narishige International, East Meadow, NY) at room temperature in FHM containing 0.01% (v/w) polyvinyl alcohol. The siRNA was diluted in PBS to a final concentration of 1  $\mu$ g/ $\mu$ L, and 4 to 5 pL siRNA solution was injected into the cytoplasm of each 1-cell embryo (zygote). In control groups, 4 to

5 pL PBS or nonfunctional siRNA (Invitrogen) at 1  $\mu$ g/ $\mu$ L in PBS was microinjected into each pronuclear-stage embryo.

**ICNI.** We previously have described<sup>10</sup> the ICNI procedure to derive cloned mouse embryos. Briefly, 6- to 8-wk-old B6D2F1 female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotrophin, followed 48 h later by injection of 5 IU human chorionic gonadotrophin. Oocyte–cumulus complexes were collected and incubated briefly in warm FHM containing 300 U/mL bovine testis hyaluronidase to remove cumulus cells. Oocytes then were cultured in microdrops (50  $\mu$ L each) of KSOM, covered with mineral oil, and placed under 5% CO<sub>2</sub> in humidified air at 37.5 °C prior to enucleation. Enucleation was performed in FHM containing 5  $\mu$ g/mL cytochalasin B. Cumulus cells were used as nuclear donor cells, which were stored in FHM at 4 °C before use. Nuclei of cumulus cells were isolated in BSA-free FHM containing 0.01% (v/w) polyvinyl alcohol by repeated aspiration into and ejection from a micropipette (diameter, 5 to 6  $\mu$ m). After collection, a single nucleus was injected into the cytoplasm of an enucleated oocyte that had been cultured in KSOM for as long as 2 h after enucleation. Reconstructed oocytes were activated by culture in Ca<sup>2+</sup>-free CZB medium (EmbryoMax, Millipore, Burlington, MA) containing 10 mM SrCl<sub>2</sub> and 5  $\mu$ g/mL cytochalasin B for 6 h at 37 °C and then collected into KSOM.

**Embryo culture.** After injection of siRNA or cloning, embryos were incubated and cultured in 5% CO<sub>2</sub> and humidified air at 37.5 °C in KSOM until late-blastocyst stage (4.5 d post coitus). Embryo development to 2-cell, 4-cell, morula, and blastocyst stages was recorded every 12 h. For evaluation of gene expression and development, samples of 25 embryos per treatment were harvested at random from each group (described later). Five replicate trials were conducted for each treatment.

**Immunofluorescent labeling.** By using monoclonal mouse antibodies to EGF, TGF $\alpha$  and EGFR (UniTech ABC kit, EMD Biosciences, San Diego, CA), these growth factors underwent immunofluorescent labeling to assess their expression levels as previously described.<sup>10</sup> Briefly, embryos were selected randomly and fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton 100, and incubated with the appropriate primary monoclonal antibody conjugated with fluorescein isothiocyanate. In the combined treatment (EGF and TGF $\alpha$ ) group, the primary antibody (5  $\mu$ g/mL, Calbiochem, San Diego, CA) to EGF was conjugated with fluorescein isothiocyanate, whereas that to TGF $\alpha$  was conjugated with phycoerythrin. Negative controls were established concurrently by using PBS containing 2% BSA instead of the primary antibody. All images (zygotes and embryos) were captured under a 40 $\times$  objective (magnification,  $\times$ 630; resolution, 1300  $\times$  1030 pixels at 150 pixels per inch) by using the same exposure for all images. Fluorescence intensity was measured (Image-Pro Plus 4.5.1, Media Cybernetics, Bethesda, MD) by using integrated optical density. Green fluorescence of the cytoplasm or membrane was considered to indicate positive expression of the individual growth factors, whereas yellowish fluorescence indicated colocalization of EGF (green) and TGF $\alpha$  (red) in the combined treatment group. The mean value of fluorescence intensity for each embryo was corrected by subtracting the mean fluorescence of the negative control.

**Embryo cell counting.** Inner cell mass (ICM) and trophectoderm (TE) cells were differentially labeled by using fluorescent propidium iodide (red) and Hoechst 33258 (bisbenzimidazole, blue) according to published protocols.<sup>41</sup> The total numbers of ICM (blue)

and TE (pinkish red) cells were counted, and the ICM:TE ratio was calculated.

**Terminal dUTP nick-end labeling assay.** To determine apoptosis, 20 blastocysts (4.5 d post coitus) were selected randomly from each experimental group and fixed, permeabilized, and stained as previously described.<sup>5</sup> The embryos were observed under a fluorescence microscope by using a blue filter (excitation wave length, 450 to 490 nm) for dUTP-labeled nuclei and a green filter (excitation wave length, 512 to 560 nm) for cells stained with propidium iodide (red). To determine DNA fragmentation, fluorescein-isothiocyanate-labeled (yellowish) nuclei were counted as apoptotic cells, and those stained with propidium iodide were counted as intact cells. The apoptotic index was calculated by dividing the number of apoptotic cells by the total number of live cells.

**Western blotting.** For each group, 20 blastocysts were processed for immunoblotting according to the methods described previously.<sup>45</sup> Briefly, blastocysts from BSA-free KSOM were transferred into an equal volume of double-strength sodium dodecyl sulfate buffer. Proteins from each sample were resolved on 12% SDS polyacrylamide gels, transferred onto polyvinylidene fluoride membrane (Millipore), blocked with 5% BSA in TBS and 0.1% Tween 20, and incubated with the appropriate monoclonal antibody. AntiEGF and antiTGF $\alpha$  were produced by immunizing BALB/c mice with recombinant human EGF or TGF $\alpha$ , and hybridomas were derived by fusion of mouse spleen cells with P3  $\times$  63 Ag8.653 mouse myeloma cells. AntiEGFR (partially purified from human A431 carcinoma cells) was produced commercially (EMD Biosciences–Calbiochem). After incubation with goat antimouse secondary antibody (conjugated with horseradish peroxidase, EMD Biosciences–Calbiochem), immunoblots were visualized by using X-ray film.

**Statistics.** All data were analyzed by using the SPSS 11.0 statistical program (SPSS, Chicago, IL). Univariate ANOVA, Tukey honest significant difference, and Bonferroni multiple-comparison procedures for marginal means (post hoc tests), F tests, and homogeneous subsets were performed for all treatment groups. Data were expressed as mean  $\pm$  SE, and a *P* level of less than 0.05 was considered statistically significant.

## Results

**Protein expression.** Immunofluorescent labeling was performed to assess expression levels of EGF, TGF $\alpha$ , and EGFR. After treatment of embryos derived through natural mating with gene-specific siRNA, expression of EGF, TGF $\alpha$ , and EGFR were significantly decreased at all stages of development in each treatment group compared with controls (*P* < 0.01) (Table 1, Figures 1, 2 A, C). Protein expression levels after combined treatment with siRNA duplexes to EGF and TGF $\alpha$  together were not significantly different from those after treatment of EGF and TGF $\alpha$  individually (Table 1; Figure 1). In addition, expression levels after treatment with siRNAs were similar to expression levels in cloned embryos (Figures 1 and 2 A, B). Furthermore, levels of expression of EGF, TGF $\alpha$ , and EGFR were greater in late-stage than early-stage blastocysts (*P* < 0.01), although they were still significantly lower than those of control embryos at the same stage (*P* < 0.05) (Table 1, Figures 1 and 2 A, C). The effects observed were gene-specific, because expression levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase did not differ among embryos in the treatment, control, and cloned embryo groups (Figure 2 D).

**Embryo development.** We assessed the developmental competence of siRNA-treated embryos. Significantly fewer 1-cell embryos developed to blastocysts after treatment with specific siRNAs to either EGF (16.0  $\pm$  1.0 blastocysts) or TGF $\alpha$  (16.2  $\pm$  0.8 blastocysts), compared with positive (25.4  $\pm$  1.7 blastocysts) and negative (25.6  $\pm$  2.3 blastocysts) controls (*P* < 0.01 for both comparisons). These results were enhanced by treatment with siRNA to EGFR (11.8  $\pm$  1.5 blastocysts) and with siRNA to both EGF and TGF $\alpha$  simultaneously (11.4  $\pm$  1.3 blastocysts). Cloned embryos showed similar development to that of embryos derived through natural mating and treated with siRNA to either EGFR or EGF and TGF $\alpha$  together (Table 2). Embryos that failed to develop beyond the 1-cell, 2-cell, and compact morula stages were shrunken, and individual cellular nuclei were disintegrated with fragmentation of chromatin (data not shown). In contrast, surviving embryos that developed to blastocysts were not morphologically different from control embryos (Figures 1 and 3).

**Apoptosis.** To assess the influence of siRNA treatment on the apoptotic index, we determined the number of apoptotic cells in treated embryos by using terminal dUTP nick-end labeling. Apoptotic nuclei were detected in all embryos after treatment with siRNA to EGF, TGF $\alpha$ , and EGFR (Table 3, Figure 3). Compared with the mean number of apoptotic cells in control embryos (4.9  $\pm$  0.8 cells), the mean number of apoptotic cells was significantly greater after treatment with siRNA to EGF (9.0  $\pm$  0.7 cells), TGF $\alpha$  (9.2  $\pm$  0.8 cells), EGFR (11.0  $\pm$  0.9 cells), and both EGF and TGF $\alpha$  together (11.2  $\pm$  0.8 cells) (*P* < 0.01). In comparison, the mean number and percentage of apoptotic cells were similar between untreated cloned embryos and embryos derived through natural mating and treated with siRNA to either EGFR or EGF and TGF $\alpha$  together (Table 3, Figure 3).

**Embryo cell counting.** Differential labeling techniques were used to assess the total number of cells and ratio of differentiated cells in blastocysts after siRNA treatment. Compared with those in positive (4.8  $\pm$  1.0 cells) and negative (5.0  $\pm$  0.8 cells) controls, the mean total number of cells was decreased significantly after treatment with siRNA to EGF (63.0  $\pm$  2.0 cells), TGF $\alpha$  (62.8  $\pm$  2.3 cells), EGFR (58.8  $\pm$  2.1 cells), and both EGF and TGF $\alpha$  together (58.4  $\pm$  2.0 cells; *P* < 0.05 for all comparisons; Table 3, Figure 3). These results were similar to the number of cells measured in cloned embryos (63.0  $\pm$  1.6 cells). In addition, the mean ICM:TE ratio after siRNA treatment differed significantly for EGFR (1:3.10  $\pm$  0.07) and combined EGF and TGF $\alpha$  (1:3.16  $\pm$  0.04) as compared with control treatment (1:2.66  $\pm$  0.03) (*P* < 0.05). However treatment with EGF (1:2.84  $\pm$  0.09) or TGF $\alpha$  (1:2.83  $\pm$  0.08) had no significant effect. The mean ICM:TE ratio in cloned embryos (1:3.18  $\pm$  0.05) was similar to that after downregulation of EGFR and both EGF and TGF $\alpha$  expression (Table 3, Figure 3).

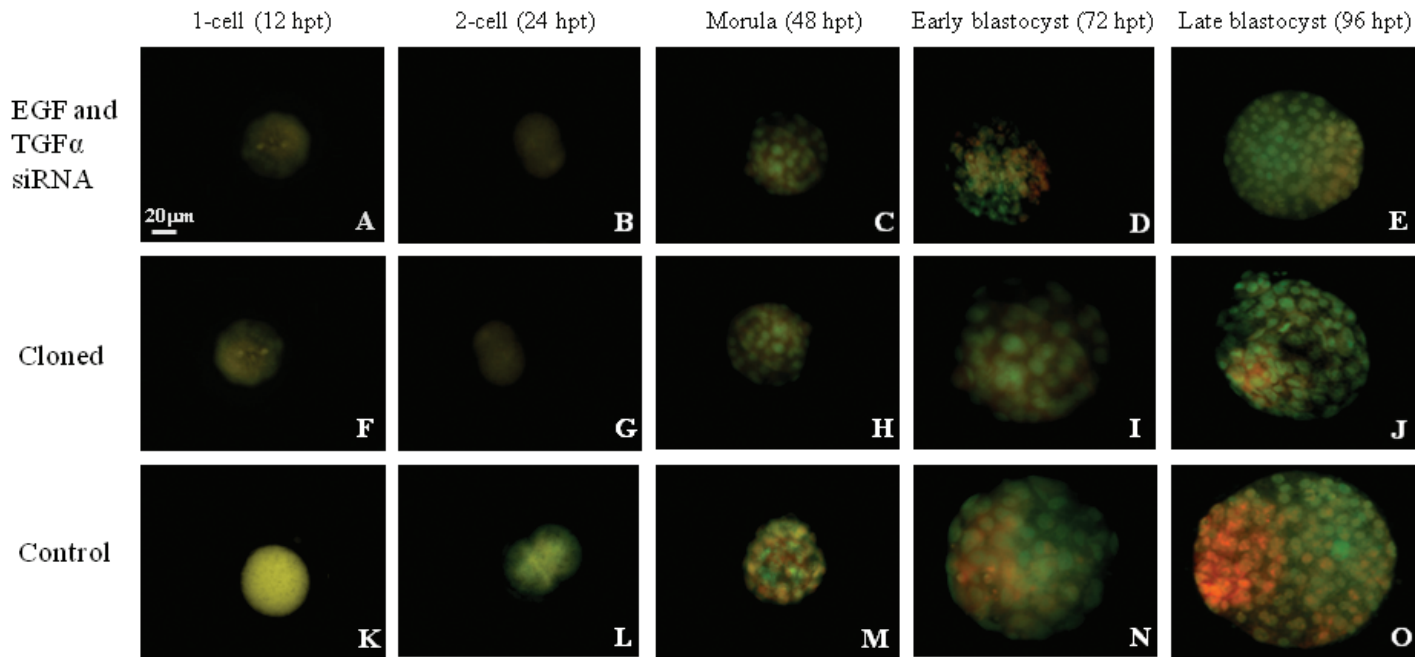
## Discussion

In the present study, we demonstrated that sequence-specific siRNAs for EGF, TGF $\alpha$ , and EGFR decreased the expression of maternally inherited and embryonically expressed genes in preimplantation mouse embryos. siRNA microinjected into the cytoplasm of 1-cell embryos, markedly inhibited the expression of growth factor genes and that of the cognate receptor immediately after transfection. Peak suppression of gene expression occurred at the 2-cell stage and decreased gradually yet progressively until returning to control levels by the late-blastocyst stage. These results indicate that siRNA-mediated RNAi effectively

**Table 1.** Fluorescence intensity (lux; mean  $\pm$  SE) and percentage of expression of EGF, TGF $\alpha$ , and EGFR.

Embryonic stage	Embryos derived through natural mating						Cloned embryos Untreated
	Control treatments		siRNA treatments				
	Positive	Negative	EGF	TGF $\alpha$	EGFR	EGF + TGF $\alpha$	
1-cell	59.3 $\pm$ 0.4 <sup>a</sup> (100%)	58.9 $\pm$ 0.6 <sup>a</sup> (100%)	42.7 $\pm$ 0.5 <sup>b</sup> (72%)	41.8 $\pm$ 1.1 <sup>b</sup> (71%)	42.6 $\pm$ 0.6 <sup>b</sup> (73%)	41.2 $\pm$ 0.7 <sup>b</sup> (70%)	57.9 $\pm$ 0.9 <sup>a</sup> (98%)
2-cell	47.1 $\pm$ 0.6 <sup>b</sup> (100%)	47.2 $\pm$ 0.8 <sup>b</sup> (100%)	17.2 $\pm$ 0.7 <sup>c</sup> (36%)	16.6 $\pm$ 0.9 <sup>c</sup> (35%)	17.4 $\pm$ 0.7 <sup>c</sup> (37%)	16.3 $\pm$ 0.7 <sup>c</sup> (35%)	17.7 $\pm$ 0.6 <sup>c</sup> (38%)
Morula	67.5 $\pm$ 0.6 <sup>d</sup> (100%)	67.6 $\pm$ 0.7 <sup>d</sup> (100%)	32.1 $\pm$ 0.9 <sup>e</sup> (48%)	31.4 $\pm$ 0.6 <sup>e</sup> (46%)	31.7 $\pm$ 0.5 <sup>e</sup> (47%)	30.7 $\pm$ 0.6 <sup>e</sup> (45%)	34.5 $\pm$ 0.8 <sup>e</sup> (51%)
Early blastocyst	81.0 $\pm$ 1.0 <sup>f</sup> (100%)	80.9 $\pm$ 1.0 <sup>f</sup> (100%)	41.1 $\pm$ 0.7 <sup>b</sup> (51%)	40.5 $\pm$ 1.0 <sup>b</sup> (50%)	41.6 $\pm$ 0.7 <sup>b</sup> (51%)	39.8 $\pm$ 1.2 <sup>b</sup> (49%)	43.3 $\pm$ 1.0 <sup>b</sup> (53%)
Late blastocyst	84.4 $\pm$ 0.4 <sup>s</sup> (100%)	84.6 $\pm$ 0.5 <sup>s</sup> (100%)	67.5 $\pm$ 0.5 <sup>d</sup> (80%)	66.2 $\pm$ 0.6 <sup>d</sup> (78%)	66.8 $\pm$ 1.1 <sup>d</sup> (79%)	65.5 $\pm$ 0.6 <sup>d</sup> (77%)	64.8 $\pm$ 0.9 <sup>d</sup> (77%)

Means with different superscripts in the columns and rows are significantly different ( $P < 0.01$ ) from values for control treatments. Percentages are relative to values for control treatments (positive, transfection with non-inhibitory siRNA; negative, no transfection or injection of PBS).

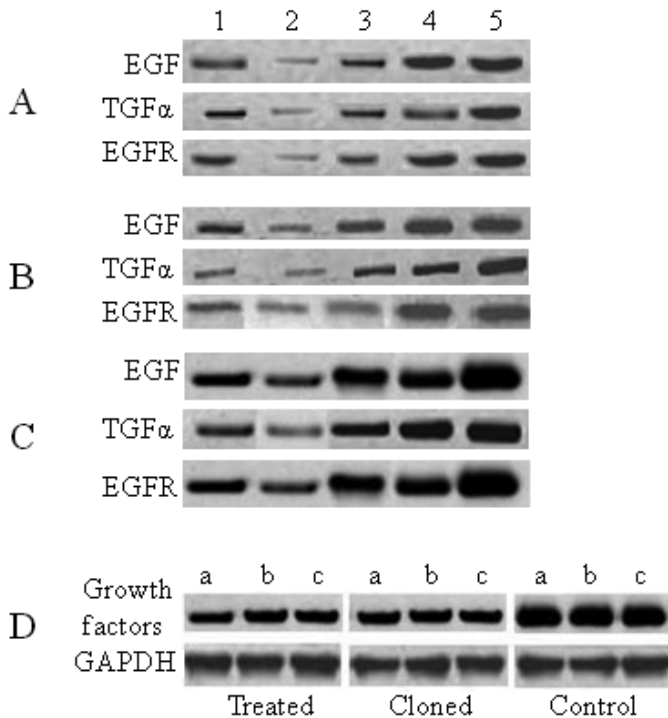


**Figure 1.** Representative immunofluorescent assay after combined (EGF, green; TGF $\alpha$ , red; colocalization, yellow) transfection of sequence-specific siRNA in 1-cell, 2-cell, morula, early blastocyst, and late blastocyst embryos harvested at 12, 24, 48, 72, and 96 h after transfection (hpt), respectively. (A through E) Transfected embryos derived from natural mating. (F through J) Untransfected, cloned embryos. (K through O) Mock-transfected embryos derived through natural mating. Magnification,  $\times 630$ .

'knocks down' both maternally inherited and embryonic gene transcripts. Previous studies have shown successful gene silencing by RNAi by using lipofection,<sup>13</sup> a vector-based approach,<sup>7,31</sup> electroporation,<sup>8,18</sup> and by microinjection.<sup>38</sup> Further, our results are consistent with previous studies that RNAi transiently yet effectively inhibited mRNAs recruited during oocyte maturation as well as embryonically expressed genes in early mouse embryos.<sup>21,39</sup>

In the present study, the observed differences in downregulation of gene expression possibly were due to a switch from maternal to embryonic expression and to the instability of mRNA, particularly at the transition between the 1- and 2-cell stages. The degradation of specific transcripts of maternal mRNA was already underway in 1-cell embryos and maximized by the 2-cell

stage. These findings support previous results suggesting that early cleavage-stage embryos rely on maternally synthesized proteins from mRNA during oogenesis<sup>23</sup> before embryonic expression initiates. Toward the end of the 2-cell stage, the first wave of gene activation (zygote genome activation)<sup>26,33,34</sup> occurs and maternal mRNA continues to degrade as embryonic mRNA transcription and protein synthesis take over.<sup>43</sup> The second wave of embryonic gene activation (midpreimplantation genome activation) precedes the morula stage.<sup>19,20,29</sup> The slow recovery of gene expression in late blastocyst that followed midpreimplantation genome activation perhaps indicates increases in the numbers of endogenous embryonic transcripts and transient downregulation of growth factors gene expression due to degradation of siRNA. These effects of siRNA treatment were highly gene-specific, in



**Figure 2.** Immunoblotting. EGF, TGF $\alpha$ , and EGFR protein expression after treatment with siRNA. (A) Embryos derived from natural mating. (B) Untransfected, cloned embryos. (C) Mock-transfected embryos derived through natural mating. Lanes: 1, 1-cell; 2, 2-cell; 3, morula; 4, early blastocyst; 5, late blastocyst. (D) Expression of glyceraldehyde-3-phosphate dehydrogenase after treatment of embryos derived through natural mating or cloned embryos with siRNA to EGF (lane a), TGF $\alpha$  (lane b), and EGFR (lane c) and mock-transfected embryos derived through natural mating. All embryos harvested at late blastocyst stage. Magnification,  $\times 630$ .

that expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase never varied among groups.

In comparison, gene expression of *EGF*, *TGF $\alpha$* , and *EGFR* after treatment with siRNAs specific to those genes was similar between ICNI-derived, cloned mouse embryos and those due to natural mating. In our previous study,<sup>10</sup> we compared the pattern, timing, and level of expression of these same growth factors in ICNI-derived cloned embryos and those of embryos derived in vivo and embryos fertilized in vitro. We found that expression of growth factors occurred first in 1-cell stage embryos and then steadily decreased in 2-cell stage embryos before progressively increasing in compact morula and blastocysts, similar to findings from in-vivo-derived and in-vitro-fertilized embryos. However, expression levels of *EGF*, *TGF $\alpha$* , and *EGFR* in cloned mouse embryos were less than in embryos generated either by natural mating or in vitro fertilization. Therefore, the accumulated evidence suggests that incomplete or inappropriate epigenetic reprogramming of donor nuclei likely is the primary cause of failures in cloning<sup>36</sup> and alters the expression of various genes and transcription factors, such as *H19*, *IGF2R*, and *Oct4* in cloned embryos.<sup>3</sup>

Although allele-specific imprints are resistant to genomic-wide demethylation during preimplantation development and imprinted genes of somatic nuclei remain largely unaffected,<sup>42</sup> some instances do exist in which imprinted genes are affected and lead to fetal and placenta growth anomalies.<sup>37</sup> The change in expres-

sion is associated with a loss of DNA methylation involved in the regulation of gene imprinting.<sup>46</sup> Similarly, recent studies support our previous studies<sup>10</sup> that cloned preimplantation mouse embryos show correct timing but altered levels of gene expression and suggest that genome reprogramming is incomplete.<sup>35</sup> In contrast, successful cloning can be expected to occur through appropriate recapitulation of a normal embryonic pattern of gene expression.<sup>29</sup> Further, failure to express any of the imprinted genes indicates that epigenetic information associated with imprinted genes is not faithfully retained in cloned embryos.

In the present study, we demonstrated that downregulation of *EGF*, *TGF $\alpha$* , and *EGFR* markedly increased apoptosis, decreased the embryo development rate and the numbers and ratios of ICM and TE cells, and negatively affected preimplantation embryo development and survival. These results support our hypothesis that RNAi acts in the mouse by either inducing degradation of targeted mRNA or inhibiting its translation.<sup>31</sup> Either way, the consequence of these effects is the disruption of normal control and regulation of the expression of targeted genes. In comparison, the changes in the ICNI-derived cloned mouse embryos were very similar to those in embryos derived by natural mating after ablation of *EGF*, *TGF $\alpha$* , and *EGFR* expression. These results are consistent with our earlier study,<sup>10</sup> in which cloned mouse embryos showed decreases 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 consistent with those of naturally derived embryos after combined treatment, with immunoneutralizing antibodies to *EGF* and *TGF $\alpha$* .

Our earlier study tested whether in vitro supplementation of exogenous *EGF* and *TGF $\alpha$*  has beneficial effects on the development of cloned embryos.<sup>12</sup> Those results demonstrated that supplementation with mitogenic growth factors significantly increased blastocyst formation, the total number of cells, and the ICM:TE cell ratio in cloned embryos. Therefore, our previous results combined with those from the present study indicate that mitogenic growth factors play key regulatory roles in cell proliferation and differentiation.

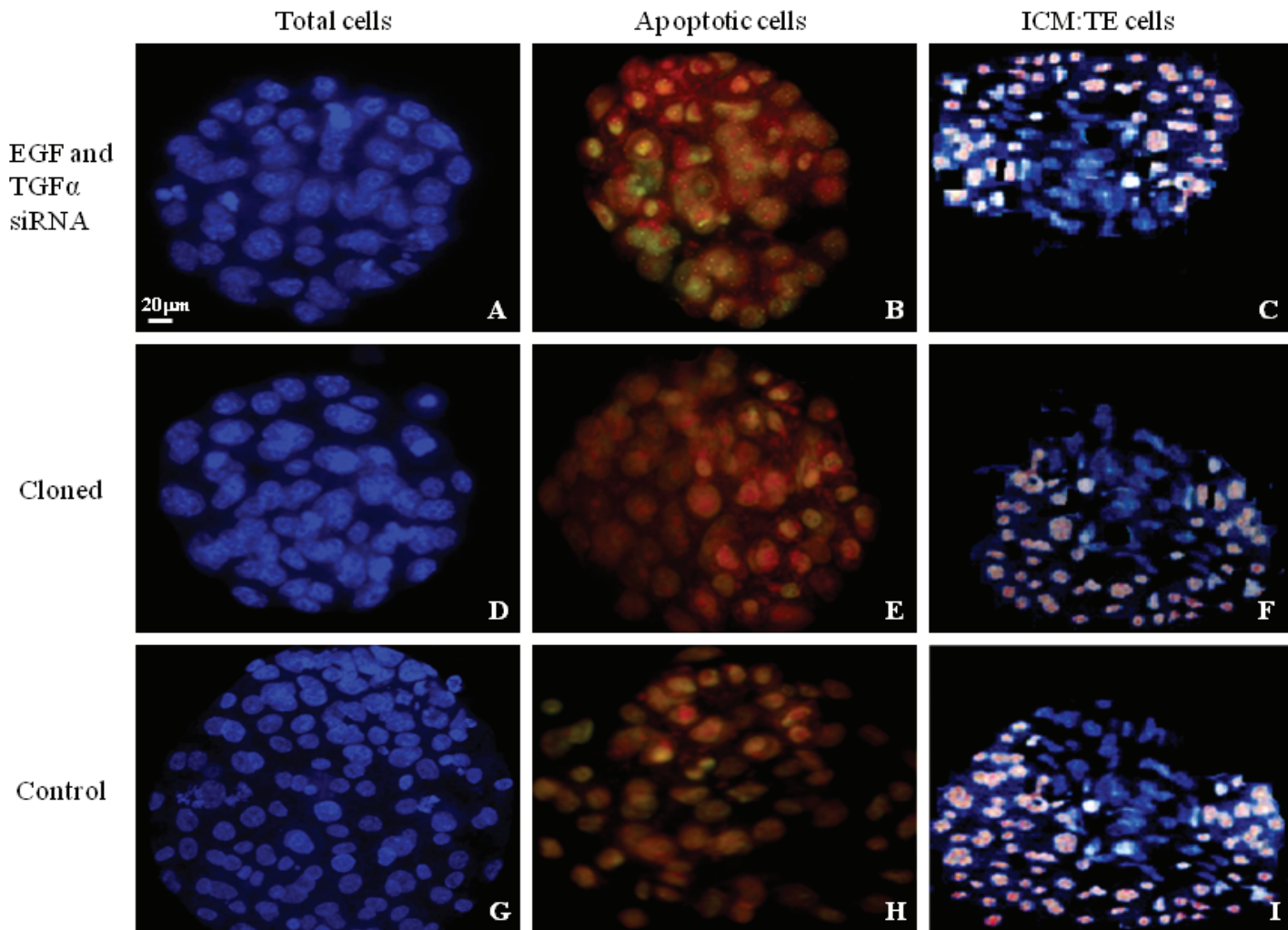
Delay or interruption of key genes involved in cell growth and differentiation ultimately activates programmed cell death.<sup>34</sup> This observation is supported further by other studies indicating that maternally inherited and embryonically expressed mitogenic growth factors play crucial stimulatory and regulatory roles in oocyte maturation and the development of preimplantation embryo.<sup>1,20</sup> In particular, *EGF* and *TGF $\alpha$*  are thought to play key regulatory roles in the development of preimplantation embryos by promoting nuclear and cytoplasmic maturation, optimizing cellular cleavage, and synchronizing embryonic and maternal maturation to ensure successful implantation.<sup>3,13,22</sup> Thus *EGF* and *TGF $\alpha$*  are implicated as important factors for embryo survival.<sup>3,11</sup>

We conclude that siRNA efficiently downregulates maternally inherited and embryonically expressed endogenous *EGF* and *TGF $\alpha$*  and their receptor, *EGFR*. Gene downregulation through RNAi decreases gene expression, thus impairing survival and delaying preimplantation embryo development. Therefore, by nature of its specificity and the timing of inhibition, posttranscriptional gene silencing is an efficient and appropriate tool not only for functional studies of individual genes but also for genome-wide screening. Dysregulation of the expression of these growth factors may explain, at least in part, the poor developmental potential of cloned mammalian embryos. The present study con-

**Table 2.** Development of 1-cell embryos to blastocysts

Embryos	No. of embryos (mean ± SE)					
	Controls		siRNA treatments			
	Positive	Negative	EGF	TGF- $\alpha$	EGF-R	EGF + TGF- $\alpha$
Natural mating						
1-cell	29.4 ± 2.0	29.8 ± 1.9	29.2 ± 1.6	30.0 ± 1.5	28.6 ± 1.6	30.0 ± 2.0
Blastocyst	25.4 ± 1.7 <sup>a</sup> (86%)	25.6 ± 2.3 <sup>a</sup> (86%)	16.0 ± 1.0 <sup>b</sup> (55%)*	16.2 ± 0.8 <sup>b</sup> (54%)*	11.8 ± 1.5 <sup>c</sup> (38%) <sup>†</sup>	11.4 ± 1.3 <sup>c</sup> (38%) <sup>†</sup>
Cloned						
1-cell	not done	28.3 ± 1.3	not done	not done	not done	not done
Blastocyst	not done	10.0 ± 0.3 (35%)	not done	not done	not done	not done

Means with different superscripts in the columns and rows are significantly different ( $P < 0.01$ ) from control treatments. Percentages are relative to values for control treatments (positive, transfection with non-inhibitory siRNA; negative, no transfection or injection of PBS). \* ( $P < 0.05$ ) and <sup>†</sup>( $P < 0.01$ ) indicate significant differences from control treatments.



**Figure 3.** Representative immunofluorescent assay of blastocysts after combined transfection of embryos with siRNA to *EGF* and *TGF $\alpha$* . All cells, blue; apoptotic cells, yellow; ratio of ICM (blue):TE cells (pink to lavender), respectively. (A through C) Transfected embryos derived through natural mating. (D through F), Untreated cloned embryos. (G through I) Mock-transfected embryos derived through natural mating. Magnification,  $\times 630$ .

firm our earlier work,<sup>12</sup> in which in vitro supplementation of growth factors improved the maturation and survival of cloned embryos. Further, our previous findings combined with the pres-

ent results suggest that mitogenic growth factors can support nuclear reprogramming and enhance cloning efficiency in mice.

Table 3. Assessment of embryos (mean  $\pm$  SE)

Embryos	Assessment	No. of embryos (mean $\pm$ SE)					
		Controls		siRNA treatments			
		Positive	Negative	EGF	TGF- $\alpha$	EGF-R	EGF + TGF- $\alpha$
Natural mating	Apoptosis	4.8 $\pm$ 1.0 (6%)	5.0 $\pm$ 0.8 (6%)	9.0 $\pm$ 0.7 <sup>b</sup> (14%)*	9.2 $\pm$ 0.8 <sup>b</sup> (15%)*	11.0 $\pm$ 0.9 <sup>c</sup> (19%)*	11.2 $\pm$ 0.8 <sup>c</sup> (19%)*
	Cell counting	74.2 $\pm$ 1.4 (100%)	74.5 $\pm$ 2.3 (100%)	63.0 $\pm$ 2.0 <sup>b</sup> (84%)*	62.8 $\pm$ 2.3 <sup>b</sup> (84%)*	58.8 $\pm$ 2.1 <sup>b</sup> (79%)*	58.4 $\pm$ 2.0 <sup>b</sup> (78%)*
	ICM:TE ratio	1:2.67 $\pm$ 0.05	1:2.66 $\pm$ 0.03	1:2.84 $\pm$ 0.09 <sup>a</sup>	1:2.83 $\pm$ 0.08 <sup>a</sup>	1:3.10 $\pm$ 0.07 <sup>b,*</sup>	1:3.16 $\pm$ 0.04 <sup>b,*</sup>
Cloned	Apoptosis	not done	11.2 $\pm$ 0.4 (18%)	not done	not done	not done	not done
	Cell counting	not done	62.8 $\pm$ 2.0 (84%)	not done	not done	not done	not done
	ICM:TE ratio	not done	1:3.18 $\pm$ 0.05	not done	not done	not done	not done

Means with different superscripts in the columns and rows are significantly different ( $P < 0.01$ ) from control treatments. Percentages are relative to values for control treatments (positive, transfection with non-inhibitory siRNA; negative, no transfection or injection of PBS). \* ( $P < 0.05$ ) and † ( $P < 0.01$ ) indicate significant differences from values from control treatments.

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