9-30-1988

Developmental Expression of PDGF, TGF-a, and TGF-j Genes in Preimplantation Mouse Embryos

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Science **241**, 1823 (1988);
DOI: 10.1126/science.3175624

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

Escherichia coli strain in which the synthesis of IL-3 was driven by the bacteriophage λ pL promoter. The intercellularly expressed IL-3 was purified to homogeneity by reversed-phase high-performance liquid chromatography in the presence of urea and dithiothreitol. The biologically active protein was produced by dilution of the denaturant, addition of oxidized glutathione, and dialysis against decreasing concentrations of urea. The purified IL-3 was formulated in physiological saline, and the final preparation had less than 1 U of endotoxin per milligram as measured by the *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD).

22. We thank R. Kamen and P. Mayer for many useful discussions, J. Witek-Giannotti and A. Ciarletta for expert technical assistance, R. Steininger and the Genetics Institute Pilot Development Laboratory for providing the human GM-CSF and IL-3, and P. Holding for assistance in preparing the manuscript. Supported in part by Sandoz, Genetics Institute, and NIH grant RR00168 awarded to the New England Regional Primate Research Center.

14 April 1988; accepted 19 July 1988

Developmental Expression of PDGF, TGF-α, and TGF-β Genes in Preimplantation Mouse Embryos

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Control of growth and differentiation during mammalian embryogenesis may be regulated by growth factors from embryonic or maternal sources. With the use of single-cell messenger RNA phenotyping, the simultaneous expression of growth factor transcripts in single or small numbers of preimplantation mouse embryos was examined. Transcripts for platelet-derived growth factor A chain (PDGF-A), transforming growth factor (TGF)–α, and TGF-β1, but not for four other growth factors, were found in whole blastocysts. TGF-α, TGF-β1, and PDGF antigens were detected in blastocysts by immunocytochemistry. Both PDGF-A and TGF-α were detected as maternal transcripts in the unfertilized ovulated oocyte, and again in blastocysts. TGF-β1 transcripts appeared only after fertilization. The expression of a subset of growth factors in mouse blastocysts suggests a role for these factors in the growth and differentiation of early mammalian embryos.

BECAUSE PREIMPLANTATION MOUSE embryos grow and differentiate in the absence of exogenous factors, endogenous factors must sustain the embryo during the first seven to eight cleavage divisions (*1*). Indirect evidence indicates that preimplantation embryos may make growth factors. Cultured embryos from around the time of implantation produce transforming growth factor–like bioactivity that promotes anchorage-independent growth (*2*). Teratocarcinoma cells, which are thought to be similar to primitive ectoderm, produce PDGF (*3*), insulin-like growth factor–II (IGF-II) (*4*), and three stem cell growth factors (*5*). After implantation in the uterus, mouse embryos produce the fibroblast growth factor homolog *int-2*, TGF-α, TGF-β (*6*), and IGF-II (*4*), but the presence of these growth factors cannot be extrapolated to the preimplantation embryo. Direct evidence for growth factor transcripts of low copy number has been impossible to obtain in preimplantation embryos, because thousands of embryos are required to detect even high copy number transcripts such as histone or actin by RNA blot analysis (*7*).

We recently developed a sensitive and quantitative method for assaying the accumulation of mRNA transcripts in small numbers of cells. Our procedure, single-cell RNA phenotyping (*8*), can detect mRNA in a single cell and in ≤10 RNA transcripts, and resolves threefold differences in input over two orders of magnitude. Thus, this method overcomes the difficulties inherent in the analysis of growth factor transcripts in early embryos. It consists of a microtechnique for isolation of total RNA from 1 to 100 mouse embryos, coupled with two enzymatic steps (*9*): reverse transcription (RT) and amplification of the transcribed cDNA in a polymerase chain reaction (PCR) (*10*). The positions of the target sequences for the transcripts are shown in Table 1. Fragments were selected for inclusion of restriction sites, identification by existing cDNA clones by DNA blot analysis, and/or inclusion of introns that would generate longer fragments if DNA or unprocessed RNA contaminated the reaction. When mouse cDNA sequences were not available, oligonucleotide sequences from other species were chosen to cover areas of conserved sequence with nondegenerate amino acids on the 3′ inside ends.

We detected β-actin RNA transcripts isolated from a single mouse blastocyst by RT-PCR; the signal became stronger when the number of PCR cycles was increased from 30 to 60 (*11*). We then used RT-PCR to detect growth factor transcripts in blastocysts and determined the growth factor mRNA phenotype of the blastocysts. Three growth factor genes, TGF-α, TGF-β1, and PDGF-A, were expressed in mouse blastocysts (Fig. 1A). The blastocysts were uncultured; thus expression was not induced by prolonged handling of embryos. The cDNA

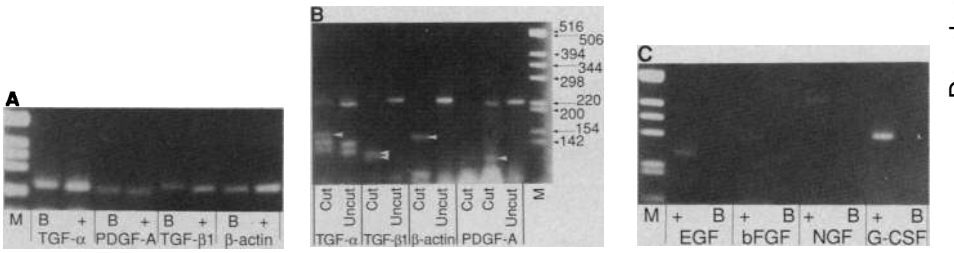

Fig. 1. Detection of mRNA transcripts in mouse blastocysts by RT-PCR. (**A**) Expression of three growth factor transcripts in mouse blastocysts demonstrated by agarose gel electrophoresis of the RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR reaction mixtures for the blastocysts (B) contained cDNA from the RNA of 2.2 embryo equivalents in each reaction, and the PCR reaction mixtures for the positive controls (+) contained cDNA from 10 ng of total RNA (*9*). The molecular size markers (M) are Hae III–digested φX174 replicative form DNA. (**B**) Restriction enzyme analysis of cDNA fragments generated by RT-PCR. Messenger RNA transcripts were expressed by blastocysts incubated with the enzymes indicated in Table 1. The diagnostic fragments (arrowheads) are TGF-α, 159 bp; TGF-β1, 119 + 125 bp; β-actin, 151 bp; and PDGF-A, 129 bp. (**C**) Lack of expression of four growth factors in mouse blastocysts demonstrated by agarose gel electrophoresis of the RT-PCR amplified reaction products obtained after 60 cycles of PCR. The PCR reaction mixtures for blastocysts (B) contained cDNA from the mRNA of 2.2 embryo equivalents in each reaction, and the PCR reaction mixtures for the positive controls (+) contained cDNA from 10 to 100 ng of total RNA. The threshold for detection of the growth factors is generally <1 to 100 pg of total RNA; for NGF, as few as ten RNA molecules can be detected by RT-PCR (*8*).

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fragments generated in the PCR were identical to those produced by adult mouse tissues known to express these transcripts. Because oligo(dT) was used to prime the RT, the fragments were generally derived from polyadenylated [poly(A)$^+$] RNA. The choice of the primers dictated that only sense-strand RNA transcripts were detected by RT-PCR. Analysis of the restriction enzyme cleavage pattern of the fragments was used to confirm their identity (Fig. 1B). We also validated the PCR reaction products by DNA blot analysis with cDNA probes (*11*). Because zygotic genome transcription is very active by the blastocyst stage (*12*), it was necessary to rule out the possibility that blastocysts express growth factor genes promiscuously. However, four other growth factors, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), nerve growth factor–β (NGF-β), and granulocyte colony-stimulating factor (G-CSF), that are expressed in tissues in adult mice were not expressed (Fig. 1C).

The accumulation of growth factor transcripts was time-dependent. PDGF-A was detected in unfertilized ovulated oocytes as a maternal transcript that disappeared between the 2- and 8-cell stages and reappeared in early cavitation blastocysts (32- to 64-cell stage) (Fig. 2A). According to dilution experiments (*11*), this change in PDGF-A expression was reflected in a more than tenfold difference in transcript numbers between oocyte and blastocyst stages. We saw a similar expression pattern for TGF-α transcripts, beginning with the unfertilized ovulated oocyte (Fig. 2B). In contrast, little TGF-β1 mRNA was present in oocytes, but this transcript appeared after fertilization and increased continuously to the blastocyst stage (Fig. 2C). As a positive control, we used the transcript for the metalloproteinase stromelysin (*13*), which is present throughout preimplantation embryonic development (*14*).

Because transcription of growth factor mRNA is not invariably coupled with the translation of these transcripts into protein (*15*), it was necessary to determine whether these transcripts were translated. We identified TGF-α (Fig. 3A), PDGF (Fig. 3B), and TGF-β1 (Fig. 3C) antigens by immunocytochemical localization in permeabilized blastocysts (*16*). The immunofluorescent signal for both TGF-α and PDGF antigens was concentrated in punctate structures in the perinuclear area of all cells of the blastocyst, and TGF-β1 antigen was found in 70 to 90% of the cells; thus the cells were probably synthesizing the growth factors. The signal was diminished by first incubating the antisera with purified soluble antigens, indicating that the signal was specific.

We have provided direct evidence that TGF-α, TGF-β1, and PDGF-A genes are expressed in mouse blastocysts, whereas EGF, bFGF, NGF-β, and G-CSF genes are not transcribed. It is possible that TGF-α, PDGF-A, and TGF-β1, individually or in combination, account for the transforming growth factor–like activity described by Rizzino (*2*).

The accumulation patterns of growth factor transcripts in mouse embryos fell into three classes. In one class, including PDGF-A and TGF-α, maternal transcripts apparently disappeared and were resynthesized in the zygote. This is also true for FGF (*17*) and PDGF-A (*18*) in *Xenopus*. In the second class, transcripts such as TGF-β1 were not present maternally but appeared as the result of zygotic transcription. In the third class, transcripts such as that for the metalloproteinase stromelysin apparently survived the breakdown of maternal mRNA that occurs after fertilization (*12*). The TGF-β–like Vg1 in *Xenopus* (*19*) is in the third class, in contrast to TGF-β1 in mouse, which is in the second class.

The presence of the three growth factor transcripts has several implications about their function in mouse preimplantation embryos. The functions of growth factors can be separated into action (effect on mitosis or differentiation) and direction (within the embryo or between the embryo and the mother). Most data on growth factor action in nonmammalian early embryos implicate their function in differentiation rather than mitosis. bFGF and TGF-β appear to be morphogens for inducing mesoderm at the

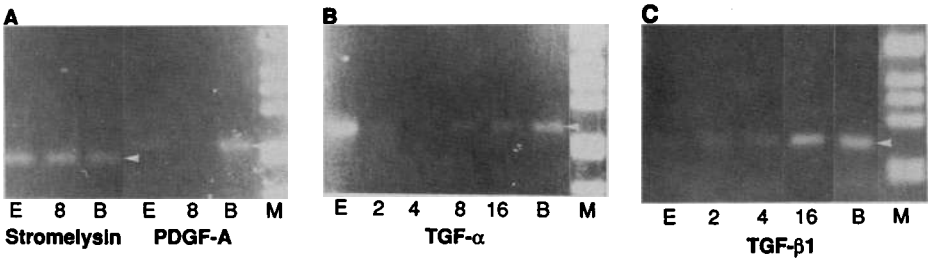

Fig. 2. Temporal regulation of growth factor gene expression in the preimplantation mouse embryo. Agarose gel electrophoresis of RT-PCR amplified reaction products obtained after 60 cycles of PCR. (**A**) PDGF-A transcripts. The PCR mixtures each contained cDNA prepared from the mRNA of the equivalent of 4.2 unfertilized ovulated oocytes (E), 6.8 eight-cell zygotes (*8*), or 3.6 early cavitation (32-cell) blastocysts (B). The total RNA used in each reaction was approximately 1.5, 4.7, and 5.3 ng, respectively (*12*). As a positive control for the RT-PCR reactions, we amplified fragments from transcripts of the metalloproteinase stromelysin, which is present at all the embryonic stages shown in the same number of embryos. (**B**) TGF-α transcripts. (**C**) TGF-β1 transcripts. In (B) and (C) the PCR mixtures contained cDNA prepared from 4 unfertilized eggs (E), 8.8 2-cell zygotes (2), 5.2 4-cell embryos (4), 2.4 16-cell morulae (16), and 4.4 blastocysts (B). In (B) 4.4 eight-cell embryos (8) were also assayed. Migration of the specific amplified fragments is indicated by arrowheads. The molecular size markers (M) are as indicated in Fig. 1.

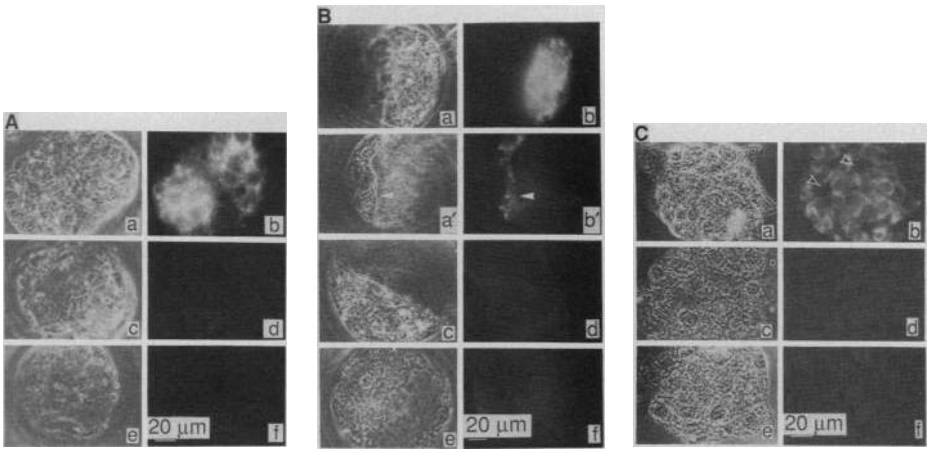

Fig. 3. Growth factor antigens in mouse blastocysts. (**A**) TGF-α. Blastocysts were treated with (a and b) anti–TGF-α; (c and d) anti–TGF-α absorbed with TGF-α antigen; (e and f) normal rabbit serum. (**B**) PDGF. (a, and a′ and b, and b′) Two different focal planes of a mouse blastocyst treated with anti-PDGF; (c and d) blastocyst treated with normal goat serum; (e and f) blastocyst treated with anti-PDGF absorbed with PDGF antigen. The arrowhead in b′ shows a mural trophectoderm cell. (**C**) TGF-β1. Blastocysts were stained with (a and b) anti–TGF-β1, (c and d) nonimmune rabbit IgG, and (e and f) anti–TGF-β1 absorbed with TGF-β1 antigen. The arrowheads in (b) show negative cells. In (A), (B), and (C) all the panels on the left are phase-contrast microscopy and all the panels on the right are immunofluorescence.

Table 1. Oligonucleotide primers used for RNA phenotyping analysis. Sequences are from published sources (*27*).

Transcript	Amplified fragment		Location of oligonucleotides in nucleotide sequence	
	Predicted size (bp)	Restriction site	5′ Oligonucleotide	3′ Oligonucleotide
β-Actin, mouse	240	Bgl II	25–44	269–245
TGF-α, rat	239	Sph I	58–78	297–277
TGF-β1, mouse	244	Sma I	1277–1296	1521–1502
PDGF-A, human	224	Acc I	622–645	848–826
EGF, mouse	350	Pst I	3953–3972	4200–4181
bFGF, bovine	282	Bam HI	295–321	578–554
NGF-β, mouse	422	Nco I	248–267	649–630
G-CSF, mouse	294	Pvu II	490–517	705–681
Stromelysin, rabbit	212	Nco I	1278–1297	1480–1461

blastulation stage in *Xenopus* (*17, 19*). However, early development of the mouse has several properties that distinguish it from that of the frog. First, the mouse egg is small, has little yolk, and quickly activates its zygotic transcription after fertilization (*12*). Second, the mouse has 10- to 12-hour cell cycle times after the first two cell cycles (*20*). These cycles have the normal G_1-S-G_2-M periods, which may allow early transcription of growth factors, as well as the opportunity to be influenced by growth factors, in contrast to the early cell cycles of the frog, which lack G_1 and G_2 (*21*).

Several lines of evidence indicate that mammalian embryonic factors are directed at maternal tissue. TGF-α and TGF-β are known to be angiogenic (*22*), and the highest density of uterine capillary beds is opposite the implanting blastocyst (*23*). In addition, the uterine environment is hypoxic (*24*), a condition that promotes production of angiogenic factors by wound healing cells (*25*). Finally, TGF-α is an EGF receptor-binding ligand, and at the time of implantation there is a surge of estrogen that increases EGF-receptor expression in the uterus severalfold (*26*). Thus, embryonic growth factors may induce the early angiogenesis and decidualization of the uterus. Whether embryonic growth factors are directed at intraembryonic targets will be determined only when it is demonstrated that genes for embryonic growth factor receptors are transcribed and translated and that these receptors are functional.

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28. We thank R. R. Adler for his participation, R. Pedersen for comments on the manuscript and for providing sabbatical facilities for R. Schultz, G. Grotendorst for the gift of anti-PDGF, N. Thompson, K. Flanders, and M. Sporn for the gift of anti–TGF-β1 and synthetic TGF-β1, M. Flannery for assistance with the immunofluorescence experiments, and R. Horiuchi and M. McKenney for preparation and editing of the manuscript. Supported by a contract from the Office of Health and Environmental Research, Department of Energy (DE-AC03-76-SF01012), NIH grants HD23539 and HD22681, a National Research Service Award (5T32 ES07106) from the National Institute of Environmental Health Sciences, and a University of California Regents Fellowship and University of California Patent Fund Award (to D.A.R.).

23 May 1988; accepted 11 August 1988