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TGFα REACTIVATES IMPRINTED *Igf2* IN THE PARTHENOGENETIC MICE EMBRYOS AND PLACENTA

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Imprinted genes play important roles in the mammalian development. In the parthenogenetic embryos (PE) there is only expression of maternally expressed genes. Therefore, PEs are appropriate experimental models to study genomic imprinting controlling mechanisms. The maternally expressed *H19* and paternally expressed *Igf2* are reciprocally imprinted genes in normal embryos. Here we studied effect of transforming growth factor alpha (TGFα) treatment in vitro (10 ng/ml at the morula stage) on the expression of *Igf2/H19* locus in mice PE (9.5-days of gestation, 25 somites) and their placentas (PP). Using RT-PCR we showed that TGFα reactivated maternally imprinted *Igf2* gene in parthenogenetic embryos and placentas. In spite of similar Tgfα expression in the pre-implantation stages, its expression in the 9.5-day parthenogenetic embryos is significantly less than in normal embryos (NE). In our experiments it was shown that reactivation of *Igf2* gene occurred independently of *H19* gene. In vitro TGFα treatment of mouse PE reactivated paternally expressed *Igf2* gene in the PE and PP. In the PE and PP both *Igf2* and *H19* were expressed. It seems that TGFα can play an important role as modulator of the *Igf2/H19* locus.

The use of parthenogenetic embryos, as experimental models, allows us to follow gene expression from maternal allele only and to get an idea about genomic imprinting controlling mechanisms. Imprinted genes are a class of genes found in placental mammals, marsupials and seed plants whose expression depends on their parental origin [1–4]. In mammals, many imprinted genes are involved in the control of fetal growth, and are expressed in both fetal and placental tissues. Recent work in mice has indicated that the roles of imprinted genes in fetal and placental tissues can be genetically separated, and that in the placenta these genes regulate both growth and specific nutrient transfer [5]. In the present time, the list of imprinted genes grows fast and more than 70 imprinted genes are now reported in the mouse [http://www.mgu.har.mrc.ac.uk/research/imprinting/imprin-viewdata.html]. Most of them are conserved in humans.

The mouse Insulin like growth factor 2 gene (*Igf2*) is located on distal chromosome 7 and closely linked with *H19* gene. *H19* and *Igf2* are reciprocally imprinted genes. The paternally expressed *Igf2* encodes a potent fetal growth factor and the maternally expressed *H19* encodes a non-coding RNA [6]. Mouse *Igf2* gene has 4 promoters (P0, P1, P2 and P3), 2 pseudo-exons and 6 exons (Acc. No. U71085). The peptide-coding region is i the exons 4–6. All transcripts are spliced to exon 4, leading to the same peptide. Promoters P1–p3 are transcribed in fetal mesodermal, endodermal and extra-embryonic tissues whereas P0 is transcribed in the placenta [7]. *Igf2* has mitogenic, morphogenic and metabolic properties [8] and plays an important role in the early stages of mammalian development. *Igf2* expression begins at the blastocyst stage of embryo development [9]. The *Igf2* gene is regulated at thae transcription and translation levels [10, 11]. In the adult mouse *Igf2* expression stops in most of the tissues with the exception of the leptomeninges and choroid plexus of the brain which express both paternal and maternal alleles [11]. Humans exhibit similar patterns of expression with one major exception, adults continue to express *IGF2* in the liver, and the maternal allele is expressed through the use of an alternate non-imprinted promoter [12, 13]. Over expression of *IGF2* has been observed in tumours of the lung, ovary, breast and liver [14].

Transforming growth factor alpha (TGFα) is a single polypeptide of 50 amino acids that is derived from a 160-amino-acid transmembrane precursor by proteolytic cleavage. TGFα is structurally and functionally related to the epidermal growth factor (EGF) family and binds to the epidermal growth factor receptor (EGFR). A possible autocrine/paracrine growth mechanism was investigated in vitro with cloned human non-small cell lung cancer (NSCLC) cell lines [15].

In this work we studied the effect of in vitro TGFα treatment on the *Igf2* gene expression in the parthenogenetic mice embryos and placentas.

Sample information and summary of results

PE + TGFα	Somites number	*Igf2*	*H19*	*TGFα*
1	25	+	+	+
2	25	+	+	+
3	25	+	+	+
4	25	+	+	+
PE – TGFa				
1	20	–	+	+
2	25	–	+	+
3	21	–	+	+
4	12	–	+	–

Note: "–": no expression "+": expression in the embryo.

MATERIALS AND METHODS

(CBA × C57BL6) F_1 mice were used for egg recovery. Parthenogenesis was done as previously described [16]. 10 ng/ml of TGFα (Sigma, USA) was added into the culture medium of parthenogenetic morulas. Parthenogenetic blastocysts were transplanted into the female uterus at the third day of pseudogestation. At 9.5-days of gestation embryos and placentas were isolated (Table).

Total RNA was isolated from normal and parthenogenetic embryos (PE) and placentas (PP) with Trizol RNA Prep 100 kit (Isogene, Russia). RT-PCR was done with GenePak™ RT-PCR Core kit (Isogene, Russia) according to manufacturer's instructions, using the following primers: *Igf2*, 5'-TCCTGTCTTCATCCTCTTCCAGCCCC-3', 5'-CGGTCCGAACAGACAAACTGAAGCGT-3' (RT-PCR product size: 198 bp); *H19*, 5'-CCACTACACTACCTGCCTCAG-3', 5'-GGTGGGTACTGGGGCAGCATTG-3' (435 bp) and 5'-CATGTCTGGGCCTTTGAA-3', 5'-TTGGCTCCAGGATGATGT-3' (245 bp) and Gapdh, 5'-ACCACAGTCCATGCCATCAC-3', 5'- TCCACCAACCCTGTTGCTGTAG-3' (452 bp). RT-PCR conditions were 40 min reverse transcription at 37°C and then 94°C, 45 s; 60°C, 30 s; 72°C, 45 s for 32 cycles, with a 2 min "hot start" at the beginning and 7 minute extension at the end. *Tgfα* RT-PCR was done with 50 ng total RNA of normal (NE) and PEs embryos using primers 5'-AGCCAGAAGAAGCAAGCCATCACT-3' and 5'-CTCATTCTCGGTGTGGGTTAGCAA-3' (RT-PCR product size 501 bp) in the conditions mentioned above, except annealing (56°C and 40 s). Primers were designed so that RNA and DNA amplification products had different sizes, and could be differentiated in the gel. Glyceraldehyde phosphate dehydrogenase gene (Gapdh) was used as internal control gene. The products were run on Ethidium bromide stained agarose or polyacrylamide gels, photographed.

RESULTS AND DISCUSSION

Serious disturbances of early development leading to the death of mammalian parthenogenetic embryos (PE) result from malfunctions of genomic imprinting, a mechanism controlling functional differences between the maternal and paternal genomes. Mouse PE usually die on the 10th day of pregnancy reaching a maximal stage of 25 somites. Exogenously added TGFα to the PE culture medium at the morula stage can modulate the effects of genomic imprinting in PE of (CBA × × C57BL/6) F_1 mice [16]. TGFα prolonged development of the parthenogenetic mice embryos to more than 12 days of gestation, whereas without TGFα parthenogenetic mice embryos died earlier [16]. On the other hand, in vitro IGF2 treatment of mouse PEs leads to a significant prolongation of their development as compared to control ones [17]. These evidences indicate that TGFα can modulate the effects of genomic imprinting in mouse PEs, which results in prolongation of their development.

Tgfα expression analysis with FISH in mouse has shown that its expression appears as soon as the 2-cell stage and increases to blastocyst stage [18]. Patterns of expression are similar in parthenogenetic and normal embryos when referred to morphological developmental stage, suggesting that no maternal imprinting exists for *Tgfα* gene [18]. In spite of similar *Tgfα* expression in the pre-implantation stages mentioned above, our RT–PCR results with initial equal concentration of total RNA showed that its expression in the later stage of development in the 9.5-day PEs is significantly less than normal embryos (Figure 1).

A comparative analysis of organospecific expression of the *Igf2* gene in 12-day-old PE and the expression of this gene in normal (fertilized) embryos at the analogous stage of morphogenesis (11th day of development) was done using in situ hybridisation with whole mount preparations of PE treated with TGFα (10 ng/ml) in vitro at the morula stage using RNA probe labelled with digoxigenine. At the morphogenesis stage under study the expression of *Igf2* gene observed in the somites, limb buds, upper part of the branchial arch, liver and gastroenteric tract rudiments of the NEs, but in the TGFα treated PEs essentially in the head (data not shown). *Igf2* expression did not observe in the untreated PEs (data not shown).

Our RT-PCR results showed that in both PEs and PPs, TGFα treatment reactivated *Igf2* expression (Figure 2 and Table). *Igf2* expression analysis using equal concentrations of total RNA in the normal and TGFα treated PEs showed that its expression was less than normal embryos but significantly more than untreated control PEs (Figure 3). It means that *Igf2* expression in the TGFα treated PEs did not result from leakage expression. *H19* expression in TGFα treated PE was less than NE and untreated PE (Figure 3). In figure 3 we used *H19* as internal control. There is *H19* expression in all of the embryos that we used, therefore

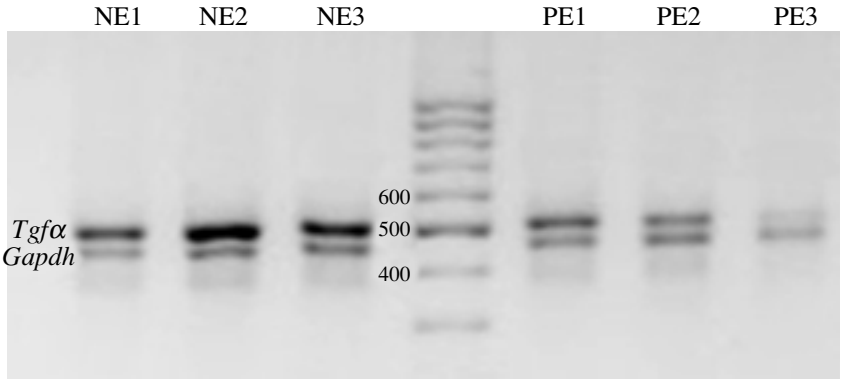

Fig. 1. *Tgfα* expression in the 9.5-days normal (NE) and control (untreated) parthenogenetic embryos (PE). Identical total RNA concentration (50 ng) was used for RT-PCR analysis.

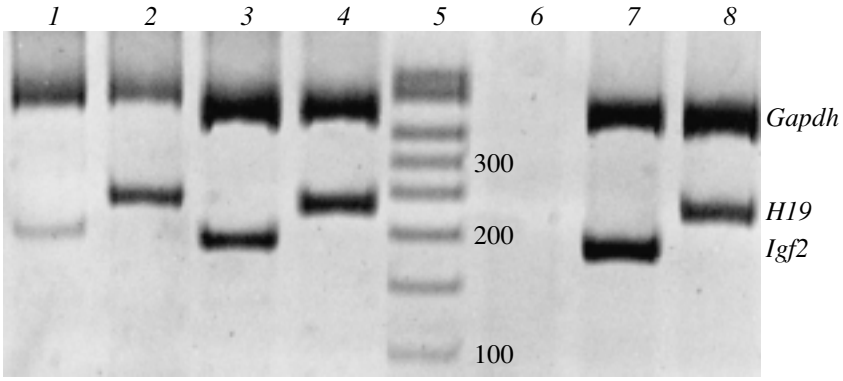

Fig.2. *Igf2* and *H19* expression in 9.5-days TGFα treated parthenogenetic embryos (PE) and placentas (PP). Results of RT-PCR analysis in polyacrylamide gel. *Igf2* (lane *1*), *H19* (lane *2*) expression in the TGFα treated parthenogenetic placenta; *Igf2* (lane *3*), *H19* (lane *4*) expression in the TGFα treated parthenogenetic embryo; marker (lane *5*); negative control, H_2O (lane *6*); *Igf2* (lane *7*), *H19* (lane *8*) expression in the normal embryo.

H19 is an appropriate internal control. We used another pair of primers for *H19*, because of interference between *Igf2* and *H19* primers and similar size of Gapdh and *H19* in this case. In fact imprinted genes repression is not complete, and in some cases the repressed allele is active to ~5% of the level produced by the expressed allele [19].

The mouse *Igf2* produces multiple transcripts during development using three different promoters. The transcripts have different 5' starting exons [20]. Differential expression analysis using RT-PCR showed that all of the embryonic *Igf2* promoters (P1-P3) were reactivated. We used a multiplex PCR for examining *Igf2* promoter usage in which cDNAs were amplified with three promoter-specific forward primers and a common reverse primer. In the PCR reaction, three promoter-specific forward primers compete with each other for the common reverse primer, depending upon the relative abundance of the promoter-derived transcripts (data not shown, primers on request).

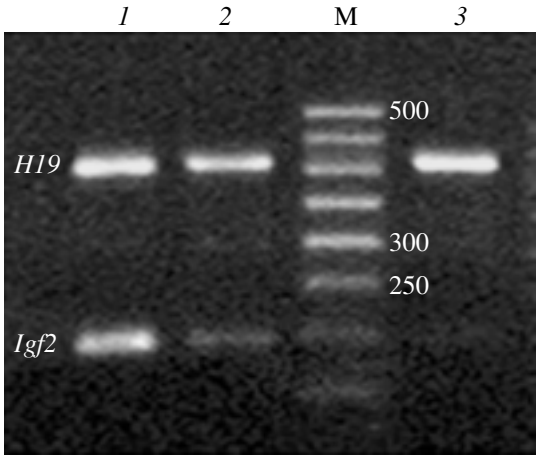

Fig. 3. *H19* and *Igf2* expression in 9.5-days control PE and TGFα treated PE. *H19* and *Igf2* expression in the mouse NE (lane *1*), TGFα treated PE (lane *2*) and control PE (lane *3*). Equal total RNA concentrations (35 ng) were used for RT-PCR analysis. *H19* is used as internal control.

Reactivation of *Igf2* gene occurred independently of *H19*. This is consistent with reports on loss of imprinting in the human genetic disorder Beckwith-Wiedmann syndrome (BWS), which can occur independently of *H19* [21, 22]. Furthermore, deletion of a repressor element located downstream of *Igf2* can lead to reactivation of normally silent maternal *Igf2* allele in the skeletal muscle independently of *H19* [23].

Reactivation of the maternally imprinted *Igf2* in TGFα induced hepatocellular carcinomas in mice was formerly reported [14]. We confirmed their results and showed that TGFα can reactivate the imprinted maternally *Igf2* in the PE and PP of mouse. It seems that TGFα exerts its effect through *Igf2* reactivation, via its mitogenic and morphogenic properties.

Kono et al. [24] recently have shown the development of a viable parthenogenetic mouse individual to adulthood from a reconstructed oocyte containing two haploid sets of maternal genome, derived from non-growing (ng) and fully grown (fg) oocytes. This development was made possible by increasing the activity of the *Igf2* gene in parthenogenetic embryos together with monoallelic expression of the *H19* gene, using mutant mice with a 13-kilobase deletion in the *H19* gene as non-growing oocyte donors. This full-term development is associated with a marked reduction in aberrantly expressed genes. Their microarray analysis of the expression of over 11000 genes showed that the expression levels of more than 1000 genes in the surviving *H19*Δ*13*-carrying parthenotes were more similar to those of normally fertilized embryos than to those of parthenotes with two intact copies of *H19*. These results show important role of *Igf2*/*H19* locus in the regulation of other genes, especially on the expression of other imprinted genes. The oligonucleotide microarray analysis also showed that in the ng*H19*Δ*13*/fgwt parthenotes, normalization nearly occurred in all of the imprinted genes analysed [24]. The results of Kono et al. show importance of *Igf2*/*H19* locus in the imprinting of other imprinted genes. Therefore, we think TGFα can play an important role in the early stages of development as a modulator of *Igf2*/*H19* locus although mechanisms of its effect need to be studied in more detail.

How does TGFα reactivate imprinted *Igf2*? Is there any role for autocrine/paracrine function of TGFα in the reactivation of the imprinted *Igf2*? These questions must be answered in the future.

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