GENE EXPRESSIONS ANALYSIS IN UTERUS AND OVARY OF MICE TREATED BY DIBUTYLTIN CHLORIDE DURING IMPLANTATION

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Introduction

Widespread use of organotin compounds in agriculture and industry has caused increasing amounts to be released into the environment. Disubstituted organotin compounds are commercially the most important derivatives, and are mainly used in the plastics industry, particularly as heat and light stabilizers. Moreover, in environment, tributyltin (TBT) is degradaded spontaneously and biochemically via a debutylation pathway to dibutyltin[1, 2]. The dietary exposure of Japanese consumers to organotin compounds was reported that daily intake was 1.7 µg/person for TBT, 0.45 µg/person for dibtyltin (DBT)[3]. The toxicity of organotins has been extensively reviewed, however, the reproductive and developmental toxicity of organotins is not well understood. Recently, we summarized the data of the studies on reproductive and developmental toxicity of organotins[4]. Both DBT and TBT caused reproductive and developmental adverse effects in experimental animals.

We previously reported that dibutyltin dichloride (DBTCl) resulted in a significant increase in the incidence of fetal malformations in rats[5]. The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of tetrabutyltin, TBT, and monobutyltin in its mode of action. Also, DBTCl[6, 7] during early pregnancy produced pregnancy failure in rats, as well as tributyltin chloride (TBTCl)[8-10]. Predominant adverse effects of DBTCl on days 0-3 of pregnancy were decrease in the pregnancy rate and increase in the incidence of pre-implantation embryonic loss. The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl[7]. Our study recently suggested that the decline in progesterone levels is primary mechanism for the implantation failure due to DBTCl[11]. To clarify the molecular mechanism of the implantation failure by DBTCl, we analyzed gene expression profiles in uterus and ovary during implantation. In this study, the mice tissues were selected for the gene expression analysis, because mice are more appropriate for the further genetic approach than rats.

At the last symposium, we reported the analyzing results of uterus gene expression on day 4 of pregnancy in the C57BL mice[12]. The results suggested that some down-regulated genes by DBTCl would be associated with the implantation failure. In that experiment, the overall gene expression levels in the untreated pregnant mice uterus had increased about 1.5 times compared to that in the untreated pseudo-pregnant mice, which indicated that the uterus in day 4 of pregnancy has already started preparation for implantation. Then, we planned analysis of effects by the DBTCl treatment in pre-implantation stage (ex. day 2 of pregnant), in terms of clarifying the triggers events for the following implantation failure. However, the pregnancy rate of the untreated C57BL mice (app. 50-60%) was considerably lower than that of the ICR mice (over than 95%). Owing to ensure the detection of the DBTCl effects in pre-implantation stage, the used strain of mice was changed to the ICR mice. Moreover, to examine the possibility of hormonal indirect effects, the analysis of ovary gene expression was added to this study.

Materials and Methods

The Crj:CD1(ICR) mice were used in this study. Daily vaginal smears were monitored from virgin female mice more than nine days before mating. On the evening of proestrus, female mice were caged overnight with untreated, proven-fertile male mice, and checked the sperm/vaginal plug for the successful mating. As for preparing the pseudopregnant mice, the vasosectomized male mice were used for the mating. The day of detecting the sperm in smear was designated to be day 0 of pregnancy. At the day 2 and 4, when were considered

to be before and around the implantation period respectively, both pregnant and pseudopregnant female mice were administered by gavage with 3.8 mg/kg of DBTCl (98% pure, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) or vehicle alone. (The experimental conditions of the eight groups were shown in the legend of the horizontal axis in Figure 1.)

At six hours after the administration, the uteruses and ovaries were dissected and dipped in the RNAlater (Ambion Inc.) for overnight at 4°C. After removing the RNAlater, the tissues were homogenized with RLT buffer (QIAGEN Inc.), and spike RNAs (non-homological Bacillus RNAs)were added for normalizing the amounts of gene expression against the sample DNA contents or the average cell number of the sample. The cDNAs were constructed from the RNAs' extracts with RNeasy kit (QIAGEN Inc.), and hybridized with the micro DNA arrays (Mouse Genome 430 2.0 Array) according to the protocol of the Gene Chip® system (Affymetrix, Inc.). The expression data were normalized with the "Percellome" normalization method[13]. The expression levels of genes were analyzed with the GeneSpring software (Silicon Genetics, Inc.).

Results and Discussion

The "Percellome" normalization method provides a "per cell" readout in mRNA copy number. The derived values reflect the absolute gene expression levels and are able to use comparing each mRNA content directly, unaffected by the varied overall expression level. The overall expression levels of day 4 uterus in pseudopregnant mice were decreased about 2 times compared to those in the pregnant mice (Fig. 1), as previously reported in the case of C57BL mice. No changes in the overall expression levels were detected in uterus of day2, and also no changes were detected in ovaries of both day 2 and day 4 (data not shown). The decrease of the overall expression suggested that the uterus of pseudopregnant in ICR mice on day 4 has started to change the uterus incapable of implantation, and also that the uterus of pregnant on day 4 has started preparation for implantation of fertilized ovum to the endometrium.

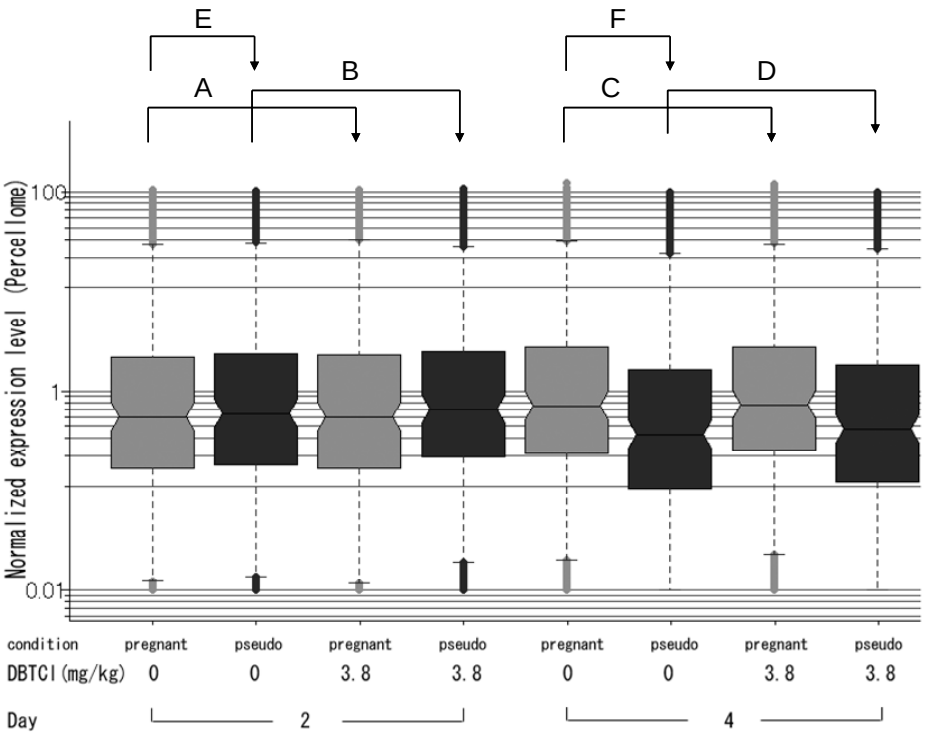

Fig. 1. The overall expression levels of uterus on pregnant day4.
(The comparison sets are labeled with characters on the arrows, which are used in the following Figures and Table.)

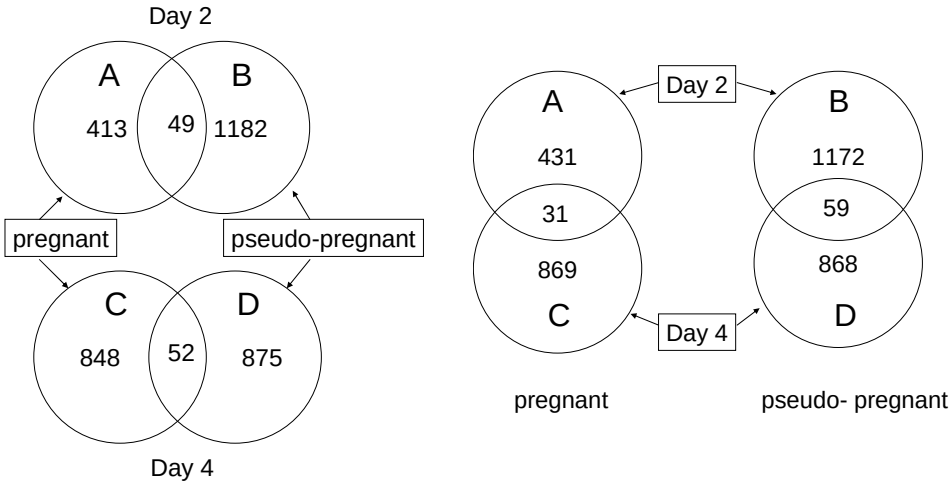

Fig.2. The Venn diagrams of the numbers of genes up-regulated more than 2 fold by DBTCl
(The characters (A, B, C, D) in the Venn diagram are corresponded to the comparison indicated in the upper of Fig.1)

In Figure 2, the Venn diagrams of the numbers of the DBTCl-dependent up-regulated genes more than two-fold were described. There were about 5 to 10 % of common up-regulated genes by the DBTCl-treatment between the arbitrary pregnant conditions. However no common gene was detected among the all pregnant conditions. Similar results were obtained from the case of down-regulated genes, and also the cases of ovaries (data not shown). These results may suggest that most of the early target genes by DBTCl in both uterus and ovary were depending on pregnancy or pseudopregnancy status and pregnant day.

Table 1. The numbers of the up- or down-regulated genes by the DBTCl-treatment among the pregnant status dependent genes

Uterus	**day2**		**day4**	
	(Comparison "**E**" in Fig.1)*		(Comparison "**F**" in Fig 1)*	
up- or down-regulated by DBTCl	> 2 fold decrease in pseudopregnant compared to pregnant (339 genes)	> 2 fold increase in pseudopregnant compared to pregnant (688 genes)	> 4 fold** decrease in pseudopregnant compared to pregnant (1594 genes)	> 2 fold increase in pseudopregnant compared to pregnant (61 genes)
> 2 fold up	0	156	4	16
> 2 fold down	77	3	146	1

Ovary	**day2**		**day4**	
up- or down-regulated by DBTCl	> 2 fold decrease in pseudopregnant compared to pregnant (475 genes)	> 2 fold increase in pseudopregnant compared to pregnant (810 genes)	> 2 fold decrease in pseudopregnant compared to pregnant (385 genes)	> 2 fold increase in pseudopregnant compared to pregnant (1098 genes)
> 2 fold up	1	282	1	381
> 2 fold down	81	1	89	1

*: The Comparison "E" or "F" is corresponded to the comparison indicated in the upper of Fig..1.

**: The threshold of decreased genes was set "4 fold", because that the overall expression levels in pseudopregnant uteri on day 4 was decreased about 2 times as those in pregnant.

In order to seek the implantation-loss associated gene, we focused the genes which were altered depending on pregnancy status. Among these pregnant status dependent genes, the numbers of the up-regulated or down-regulated genes by the DBTCl-treatment were summarized in Table 1. Interestingly, the almost of DBTCl-regulated genes among the decreased genes in pseudopregnant relative to pregnant were down-regulated. On the contrary, most of DBTCl-regulated genes among the increased genes in pseudopregnant relative to pregnant were up-regulated. These phenomenons are common to both uterus and ovary. While, these DBTCl-regulated genes included some of steroids metabolizing enzymes, kinases, and wnt signaling factors, which may be associated with hormone metabolism, cell proliferation and implantation process[14], respectively. These results suggested that almost genes regulated by DBTCl among the pregnancy status dependent genes were associated with the attenuation of pregnant (or implantation) condition, and that the common regulation mechanism exists. We are exploring the promoter regions of these genes by using the Genomatix database (Genomatix Software GmbH).

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