異常が検出されたことから、今後、分子メカニズムの解析に基づく安全性評価研究を行う必要があると考えられた。

F. 研究発表

1．論文発表

Ohtake J, Sakurai M, Hoshino Y, Tanemura K, Sato E. Expression of focal adhesion kinase in mouse cumulus-oocyte complexes, and effect of phosphorylation at Tyr397 on cumulus expansion. Mol Reprod Dev. 2015 Mar;82(3):218-31.

Shirakata Y, Hiradate Y, Inoue H, Sato E and Tanemura K, Histone h4 modification during mouse spermatogenesis. J Reprod Dev 60(5): 383-387, 2014.

Hiradate Y, Inoue H, Kobayashi N, Shirakata Y, Suzuki Y, Gotoh A, Roh SG, Uchida T, Katoh K, Yoshida M, Sato E, Tanemura K. Neurotensin enhances sperm capacitation and acrosome reaction in mice. Biol Reprod. 2014 Aug;91(2):53.1-9.

Inoue H, Hiradate Y, Shirakata Y, Kanai K, Kosaka K, Gotoh A, Fukuda Y, Nakai Y, Uchida T, Sato E and Tanemura K, Site-specific phosphorylation of Tau protein is associated with deacetylation of microtubules in mouse spermatogenic cells during meiosis. FEBS Lett 588(11): 2003-2008, 2014.

Ishikawa S, Machida R, Hiraga K, Hiradate Y, Suda Y, Tanemura K. Hanging drop monoculture for selection of optimal antioxidants during in vitro maturation of porcine oocytes. Reprod Domest Anim. 2014 Apr;49(2):e26-30.

2. 学会発表

平賀孔、種村健太郎、マウスへのネオニコチノイド系農薬アセタミプリド単回曝露による遅発中枢影響の性差、第 41 回　日本毒性学会学術年会（2014.7.）

種村健太郎、菅野 純、ネオニコチノイド系農薬による中枢神経影響解析および生殖機能影響解析、第 17 回環境ホルモン学会、第 17 回環境ホルモン学会研究発表会(2014.12.)

古川佑介、種村健太郎、相崎 健一、北嶋 聡、菅野 純、アセチルコリンエステラーゼ阻害作用をもつ殺虫剤の暴露による遅発性の中枢神経影響の比較、第 17 回環境ホルモン学会研究発表会(2014.12.10)

平賀孔、種村健太郎、マウスへのネオニコチノイド系農薬アセタミプリド単回曝露による遅発中枢影響の性差、第 17 回環境ホルモン学会研究発表会（2014.12.）

G. 知的所有権の取得状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

H26-化学-指定002

受精卵培養液中のフタル酸類の受精卵及び出生児に対する影響評価研究

分担研究者

東北大学大学院動物生殖科学分野　教授

種村健太郎

分担する研究項目

1）情動認知行動評価技術を用いたin vivo影響解析研究

2）生殖工学技術を用いたin vitro影響解析研究

分担する研究項目

1）情動認知行動評価技術を用いたin vivo影響解析研究

2）生殖工学技術を用いたin vitro影響解析研究

神経行動毒性

主たる対象は急性～亜急性の末梢影響（麻痺、痙攣）で、
遅発性の中枢影響への対応が遅れている。
（特に情動行動や認知行動）

プロファイリング的な行動影響評価のみで、
裏付けとなる科学的物証を伴わないケースが多い。

重篤な行動影響があったとしても、
神経病理所見を伴わない場合、見逃されてしまう危険がある。

胎児期-小児期における化学物質暴露影響評価が難しい。
（行動異常への関与が疑われている。）

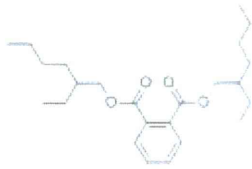
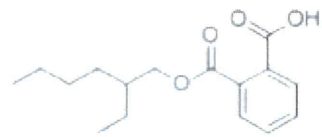
本研究班においては、
フタル酸類による情動認知行動異常を明らかにするとともに

1）それに対応する神経科学的物証を得る。

2）行動異常発現メカニズムの解明を目指す。

5

DEHP・MEHP妊娠期飲水暴露により得られた産子マウス（雄）の
情動認知行動解析

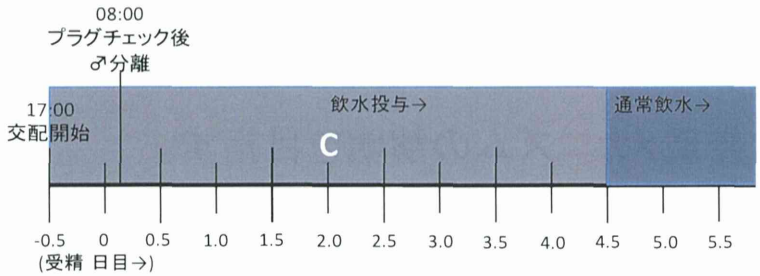
①物質名：フタル酸ビス(2-エチルヘキシル)(DEHP)	②物質名：フタル酸モノ(2-エチルヘキシル)(MEHP)
	
CAS No.：117-81-7	CAS No.：4376-20-9
分子式・分子量：$C_{24}H_{38}O_4$=390.56	分子式・分子量：$C_{16}H_{22}O_4$=278.37
性状：液体　比重：0.986	性状：液体　比重：1.07
純度：98%	純度：96%
メーカー：東京化成工業	メーカー：和光純薬株式会社
カタログ No.：P0297　Lot No：JH45M-DF	カタログ No：323-65643　Lot No：

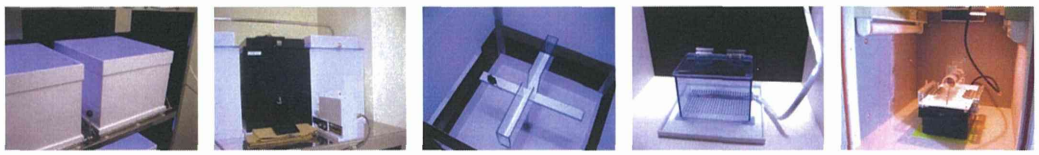
高用量(H)→20μM飲水投与
低用量(L)→2μM飲水投与

高用量(H)→50μM飲水投与
低用量(L)→5μM飲水投与

6

性周期を考慮し、1群につき♀20匹、計♀100匹♂50匹を交配する。
17時に交配及び飲水投与を開始し(PCD-0.5)、翌日AM8:00にプラグチェックを行い、
♂を分離する。4日後17時(PCD4.5)に通常飲水へ交換し、飲水投与を完了する。
その後、産まれた♂産仔を4週齢で4匹/ケージで離乳し、
体重測定の後に群分けを実施する。12週齢まで育成し、
情動認知行動バッテリー解析に供した。





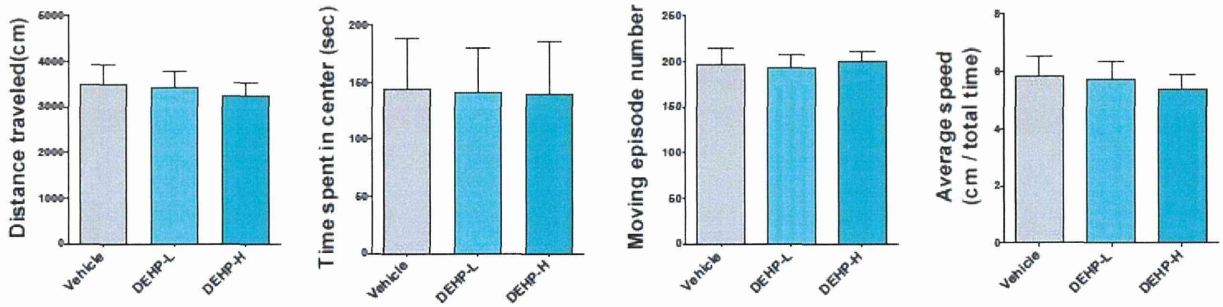
オープンフィールド試験 | 明暗往来試験 | 高架式十字迷路試験 | 条件付け学習記憶試験 | プレパルス驚愕反応抑制試験

7

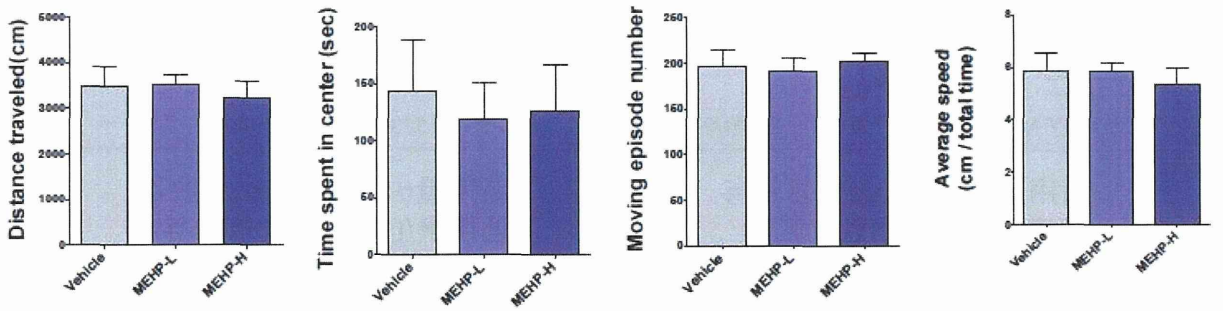
Open field test

オープンフィールド試験による
異常行動の検出は認められなかった。

DEHP



MEHP

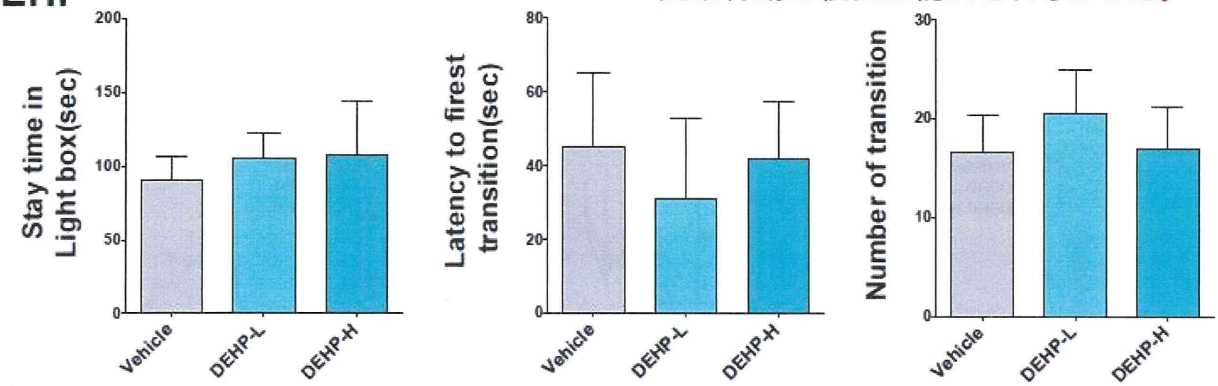


N=8, Mean ±S.D., Student's t-test

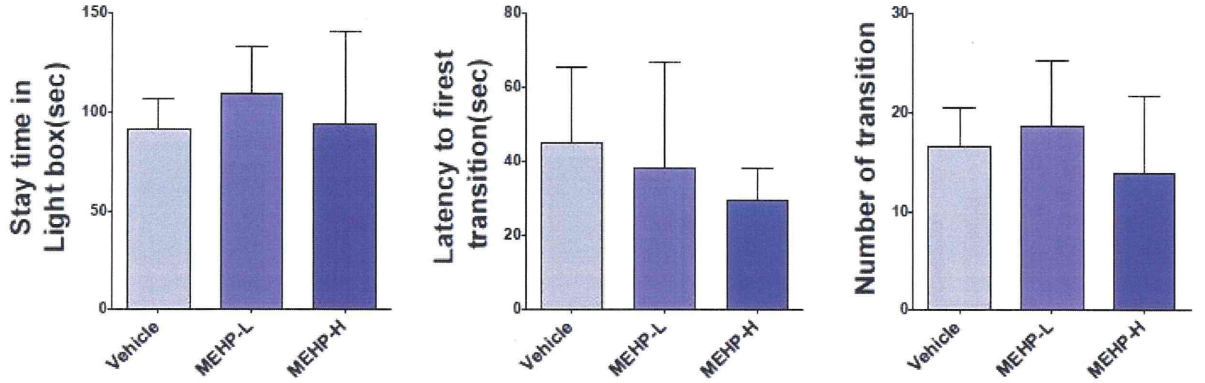
Light / dark transition test

明暗往来試験による
異常行動の検出は認められなかった。

DEHP



MEHP

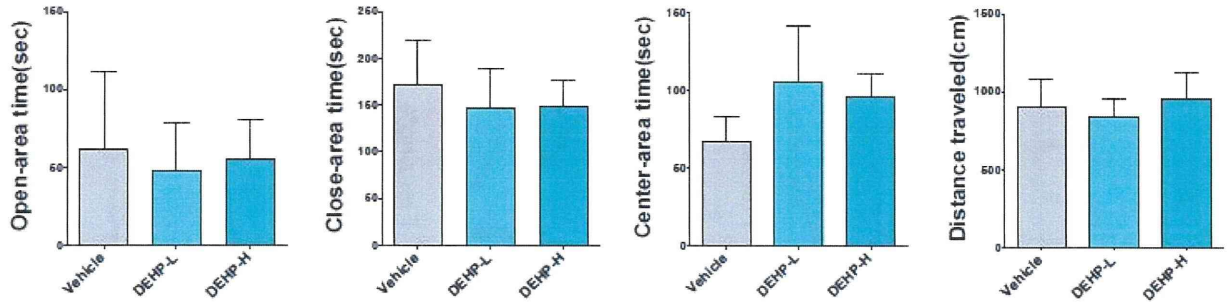


N=8, Mean ±S.D., Student's t-test

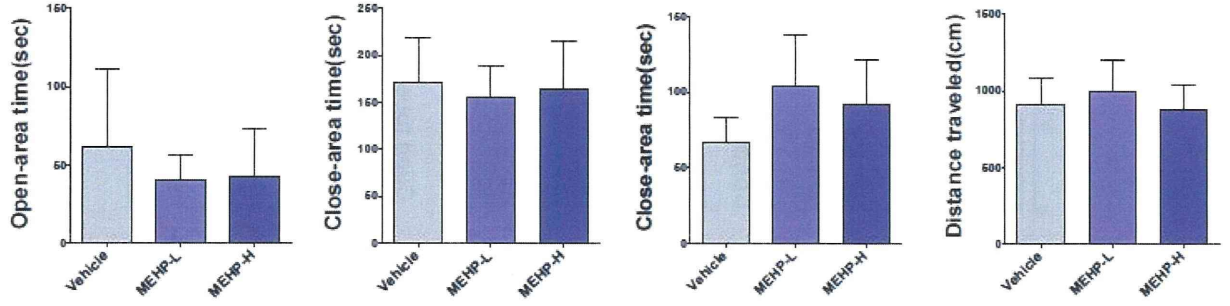
Elevated plus maze test

高架式十字迷路試験による
異常行動の検出は認められなかった。

DEHP



MEHP

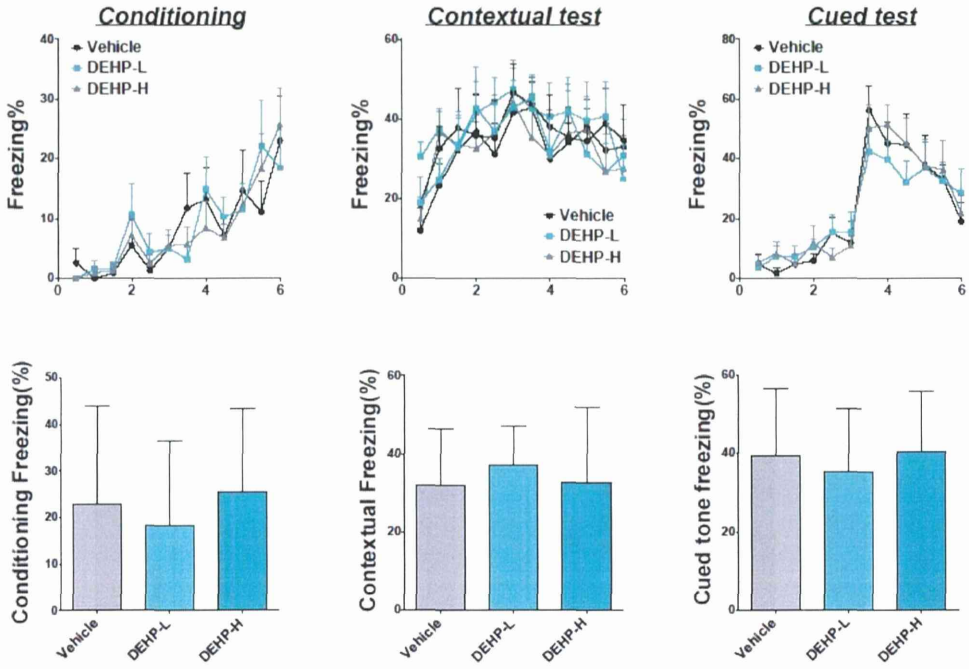


N=8, Mean ±S.D., Student's t-test

Fear Conditioning test

条件付け学習記憶試験による
異常行動の検出は認められなかった。

DEHP

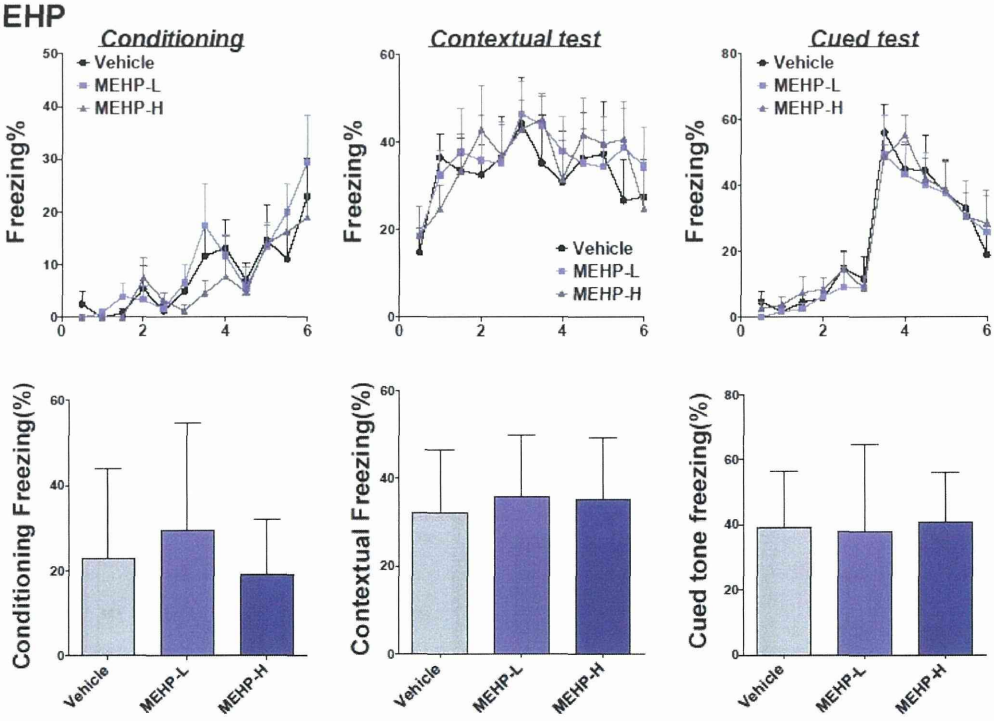


N=8, Mean ±S.D., Student's t-test

Fear Conditioning test

条件付け学習記憶試験による
異常行動の検出は認められなかった。

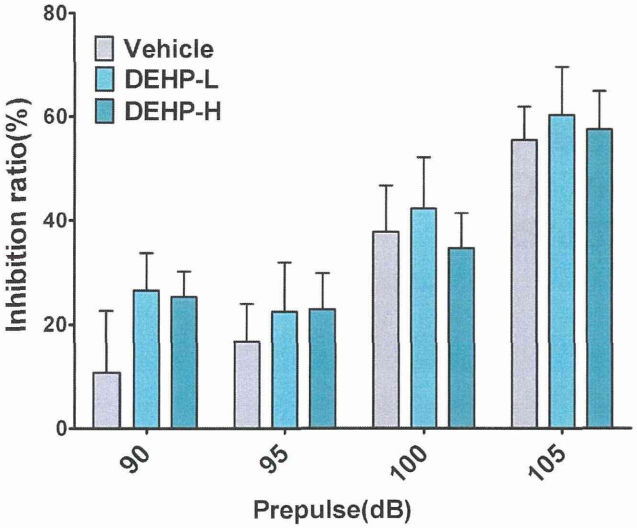
MEHP

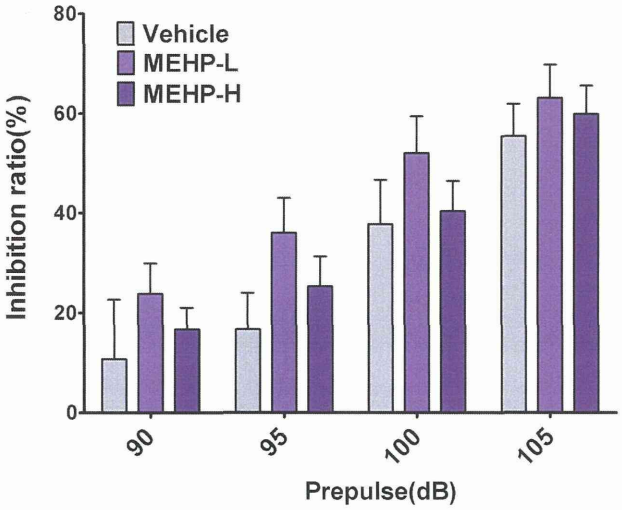


N=8, Mean ±S.D., Student's t-test

Prepulse inhibition test

プレパルス驚愕反応抑制試験による
異常行動の検出は認められなかった。





N=8, Mean ±S.D., Student's t-test

分担する研究項目

1）情動認知行動評価技術を用いたin vivo影響解析研究

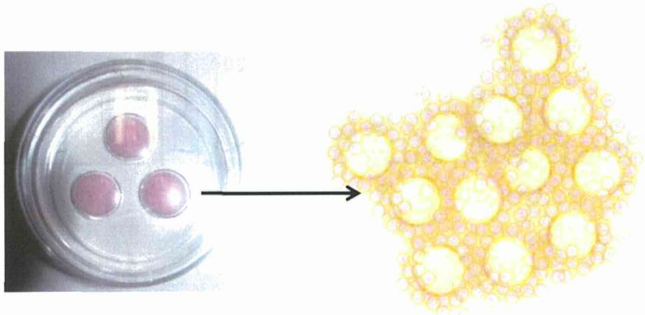
2）生殖工学技術を用いたin vitro影響解析研究

14

IVM (*in vitro* maturation)

卵巣から採取した受精能を持たない未成熟卵母細胞を
体外で培養することで成熟を促し、受精可能な状態にする。

培養シャーレ上の培養液滴内における、
複数の「卵丘細胞-卵母細胞複合体（COC）」の共培養が、
広く用いられている。



Culture Drop(CD)法

15

複数の「卵丘細胞-卵母細胞複合体（COC）」の共培養を行う上での問題点

多くの「卵丘細胞-卵母細胞複合体（COC）」が必要となる。

→実験動物を扱う上で「3Rの原則」にそぐわない。

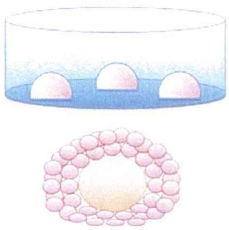
成熟不良の「卵丘細胞-卵母細胞複合体（COC）」の存在が、同一細胞液滴中の他の「卵丘細胞-卵母細胞複合体（COC）」に悪影響を及ぼす恐れが指摘される。

「卵丘細胞-卵母細胞複合体（COC）」の単独培養技術の確立が望ましい。

16

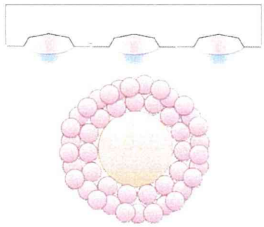
「卵丘細胞-卵母細胞複合体（COC）」
単独培養の高度化

Culture drop法



押しつぶされている状態
↓
卵丘細胞の膨化阻害・退行

吊り下げ式培養法
Hanging drop法



卵胞内に近い状態
↓
卵丘細胞の三次元的膨化の
高度化が期待できる

17

本研究班においては、
フタル酸類による成熟率影響評価とともに、

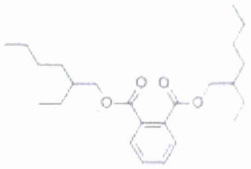
1）卵成熟過程に生じる、染色体分配影響に関わる、
関連タンパクの動態を解析する。

2）人工授精後の発生影響との関連を検討する。

18

培養液へのDEHP・MEHP添加によるマウス卵子体外培養系への影響

①物質名：フタル酸ビス(2-エチルヘキシル)(DEHP)



CAS No.：117-81-7

分子式・分子量：$C_{24}H_{38}O_4$=390.56

性状：液体　比重：0.986

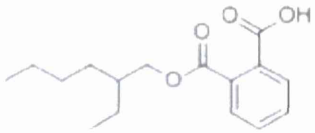
純度：98%

メーカー：東京化成工業

カタログ No.：P0297　Lot No：JH45M-DF

高用量(H)→20μM飲水投与
低用量(L)→2μM飲水投与

②物質名：フタル酸モノ(2-エチルヘキシル)(MEHP)



CAS No.：4376-20-9

分子式・分子量：$C_{16}H_{22}O_4$=278.37

性状：液体　比重：1.07

純度：96%

メーカー：和光純薬株式会社

カタログ No：323-65643　Lot No：

高用量(H)→50μM飲水投与
低用量(L)→5μM飲水投与

19

卵母細胞の採取方法

PMSG(5IU)をC57/B6マウス（4週齢）に腹腔内投与後、
46時間後に頚椎脱臼により安楽死させ、卵巣を採取した。
37℃の操作培地(0.1%polyvinyl alcohol,4mM hypoxanthineを含むLibovitz's L-15培地)
内で、26G針付きシリンジで卵胞を裂き、卵丘細胞-卵母細胞複合体(COC)を採取した。

吊り下げ式培養の方法

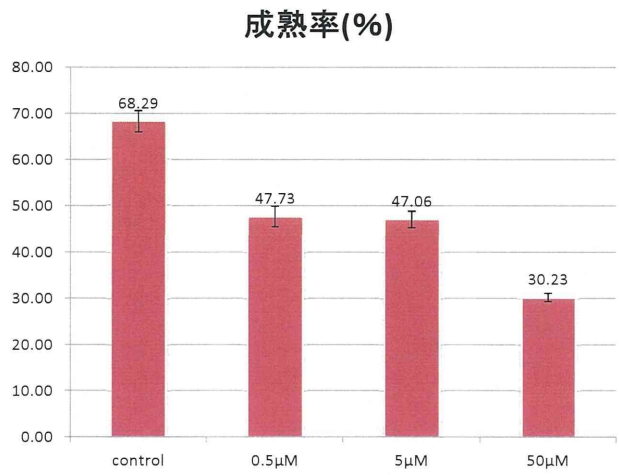
採取したCOCを、成熟培地のドロップ（100μl）に移した。
ドロップは、各群(control,0.5μM,5μM,50μM)とし、COCを洗浄後、
成熟培地(10μl)で吊り下げ式培養を行った。

紡錘体形成異常の検出方法

培養後、保存した卵母細胞(卵丘細胞は除去した)を、α-Tubulin 、核染色を行った。
その後共焦点レーザー顕微鏡を用い、紡錘体、染色体(核)の様子を観察した。

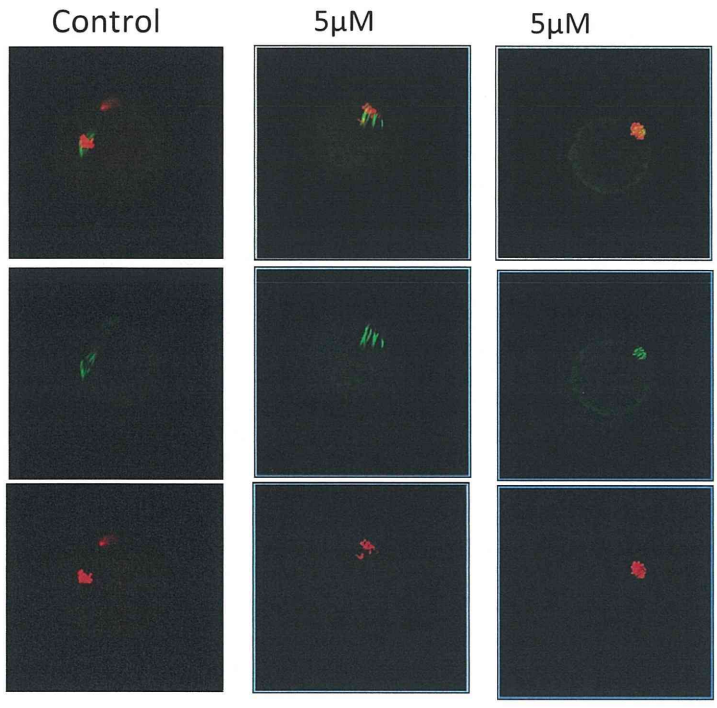
20

培養液へのMEHP添加によるマウス卵子体外培養系への影響

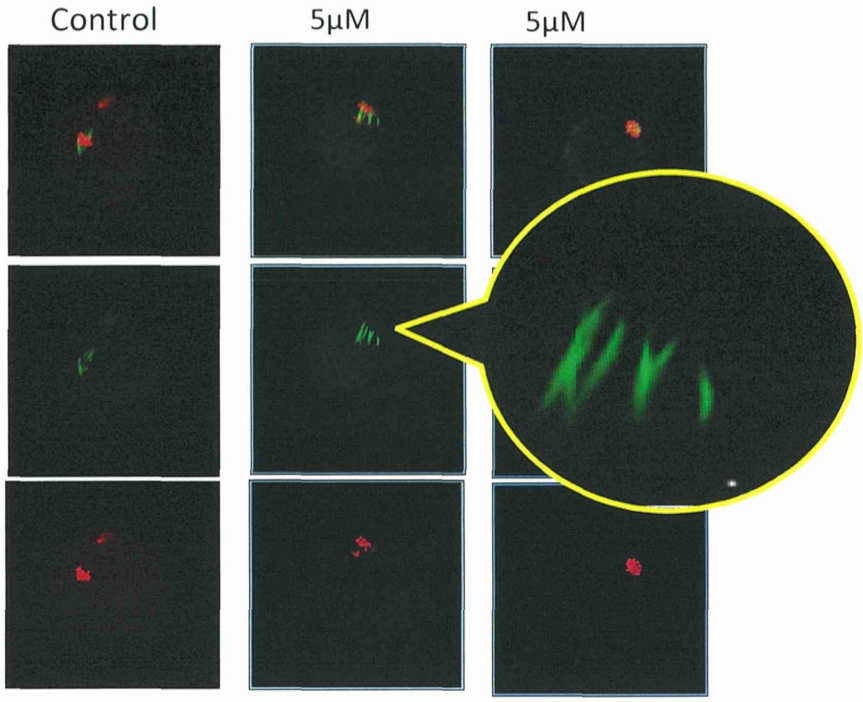


バーは標準偏差

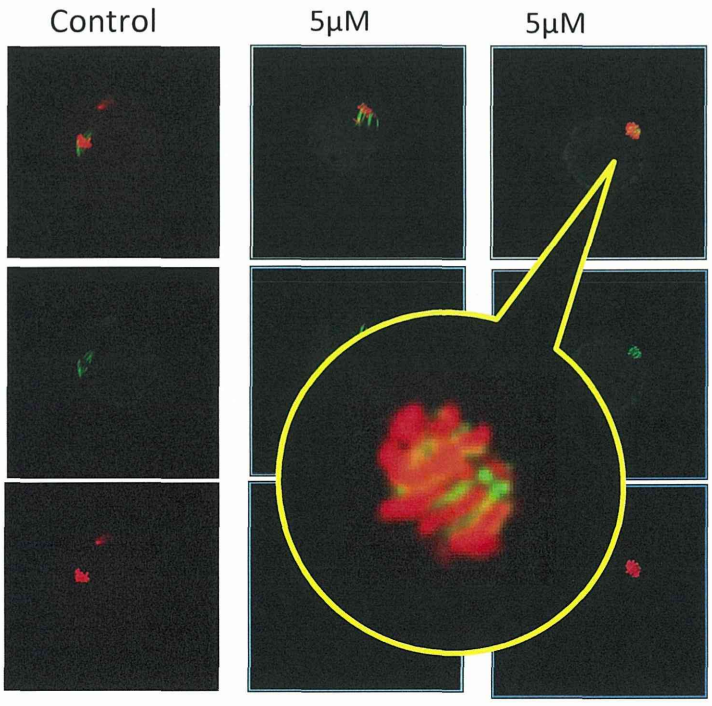
培養液へのMEHP添加によるマウス卵子体外培養系への影響



培養液へのMEHP添加によるマウス卵子体外培養系への影響



培養液へのMEHP添加によるマウス卵子体外培養系への影響



別添５

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Tanaka M, Aisaki K, Kitajima S, Igarashi K, Kanno J and Nakamura T	Gene expression response to EWS–FLI1 in mouse embryonic cartilage.	Genomics Data	2	296 - 298	2014
Tanaka M, Yamazaki Y, Kanno Y, Igarashi K, Aisaki K, Kanno J and Nakamura T	Ewing's sarcoma precursors are highly enriched in embryonic osteochondrogenic progenitors.	J Clin Invest	124 (7)	3061 - 3074	2014
Janesick A, Nguyen TT, Aisaki K, Igarashi K, Kitajima S, Chandraratna RA, Kanno J and Blumberg B	Active Repression by RARγ Signaling is Required for Vertebrate Axial Elongation.	Development	141 (11)	2260 - 2270	2014
Ohtake J, Sakurai M, Hoshino Y, Tanemura K, Sato E.	Expression of focal adhesion kinase in mouse cumulus-oocyte complexes, and effect of phosphorylation at Tyr397 on cumulus expansion.	Mol Reprod Dev.	82 (3)	218 - 31	2015
Shirakata Y, Hiradate Y, Inoue H, Sato E and Tanemura K	Histone h4 modification during mouse spermatogenesis.	J Reprod Dev	60 (5)	383 - 387	2014
Hiradate Y, Inoue H, Kobayashi N, Shirakata Y, Suzuki Y, Gotoh A, Roh SG, Uchida T, Katoh K, Yoshida M, Sato E, Tanemura K.	Neurotensin enhances sperm capacitation and acrosome reaction in mice.	Biol Reprod.	91 (2) :53	1 - 9	2014
Inoue H, Hiradate Y,	Site-specific	FEBS Lett	588	2003	2014

Shirakata Y, Kanai K, Kosaka K, Gotoh A, Fukuda Y, Nakai Y, Uchida T, Sato E and Tanemura K	phosphorylation of Tau protein is associated with deacetylation of microtubules in mouse spermatogenic cells		(11)	- 2008	
Ishikawa S, Machida R, Hiraga K, Hiradate Y, Suda Y, Tanemura K	Hanging drop monoculture for selection of optimal antioxidants during in vitro maturation of porcine oocytes.	Reprod Domest Anim.	49 (2)	26 - 30	2014

別添６

Ⅳ．研究成果の刊行物・別刷

Genomics Data 2 (2014) 296–298

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Data in Brief

Gene expression response to EWS–FLI1 in mouse embryonic cartilage

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 3 September 2014
Accepted 7 September 2014
Available online 16 September 2014

Keywords:
cDNA microarrays
Embryonic cartilage
EWS–FLI1
Ewing's sarcoma
Gene expression profiling

ABSTRACT

Ewing's sarcoma is a rare bone tumor that affects children and adolescents. We have recently succeeded to induce Ewing's sarcoma-like small round cell tumor in mice by expression of EWS–ETS fusion genes in murine embryonic osteochondrogenic progenitors. The Ewing's sarcoma precursors are enriched in embryonic superficial zone (eSZ) cells of long bone. To get insights into the mechanisms of Ewing's sarcoma development, gene expression profiles between EWS–FLI1-sensitive eSZ cells and EWS–FLI1-resistant embryonic growth plate (eGP) cells were compared using DNA microarrays. Gene expression of eSZ and eGP cells (total, 30 samples) was evaluated with or without *EWS–FLI1* expression 0, 8 or 48 h after gene transduction. Our data provide useful information for gene expression responses to fusion oncogenes in human sarcoma.

Specifications	
Organism/cell line/tissue	*Mus musculus*
Strain	BALB/c, dpc 18.5
Sex	Both male and female
Array type	Affymetrix MOE430 2.0 array
Data format	Raw data: CEL files, processed data: Excel table
Experimental factors	Tissue
Experimental features	Gene expression in eSZ cells and eGP cells with or without EWS–FLI1 expression was compared
Consent	n/a

Direct link to deposited data

Deposited data can be found here: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32618.

Experimental design, materials and methods

Preparation of mouse embryonic superficial zone (eSZ) and growth plate (eGP) cells

Femoral and humeral bones of BALB/c mouse embryos were removed aseptically on 18.5 dpc, and they were microdissected into eSZ and growth plate (eGP) under a stereomicroscope (Zeiss Stemi 2000-C, Carl Zeiss MicroImaging). Each region was minced and gently digested with 2 mg/mL of collagenase (Wako Pure Chemical) at 37 °C for 2 h. They were cultured in growth medium composed of Iscove's Modified Dulbecco's Medium (Invitrogen) supplemented with 15% fetal bovine serum, and subjected immediately to retroviral infection.

Retroviral infection

N-terminal FLAG-tagged *EWS–FLI1* was introduced into the pMYs-IRES-GFP vector. The full length *EWS–FLI1* cDNA was a kind gift from Dr. Susanne Baker. Retroviral infections of eSZ, eGP or shaft cells were performed as described [1]. Infection efficiency was examined using a FACSCalibur flow cytometer (Beckton Dickinson). Cells were harvested after fourty-eight hours of infection.

RNA isolation and microarray

GeneChip analysis was conducted to determine gene expression profiles. The per cell normalization method (Percellome method) was applied to eSZ and eGP samples [2]. Briefly, cellular lysates were prepared with RLT buffer (QIAGEN). A 10 μL aliquot of each lysate was treated with DNAse-free RNase A (Nippon Gene Inc., Japan) for 30 min at 37 °C, followed by Proteinase K (Roche Diagnostics GmbH., Germany) for 3 h at 55 °C. The aliquot was then transferred to a 96-well black plate. PicoGreen fluorescent dye (Molecular Probes Inc., USA) was added to each well, shaken for 10 s four times and then incubated for 2 min at 30 °C. DNA concentration was measured using a 96

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http://dx.doi.org/10.1016/j.gdata.2014.09.003

well fluorescence plate reader with excitation at 485 nm and emission at 538 nm. λ phage DNA (PicoGreen Kit, Molecular Probes Inc., USA) was used as standard. As reported previously [2], the grade-dosed spike cocktails (GSCs) made of the *Bacillus subtilis* RNAs corresponding to the sequences in the Affymetrix GeneChip arrays (AFFX-ThrX-3_at, AFFX-LysX-3_at, AFFX-PheX-3_at, AFFX-DapX-3_at, and AFFX-TrpnX-3_at) were prepared, and GSCs were added to the sample homogenates in proportion to their DNA concentrations. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). The GeneChip Mouse Genome 430 2.0 Array (Affymetrix) was hybridized with the cRNA generated from eSZ and eGP cells, and murine Ewing's sarcoma tissue (Table 1). After staining with streptavidin–phycoerythrin conjugates, arrays were scanned using an Affymetrix GeneChip Scanner 3000 and analyzed using Affymetrix GeneChip Command Console Software (AGCC, Affymetrix) and GeneSpring GX 11.0.2 (Agilent Technologies) as described previously [3]. The expression data for eSZ and eGP cells were converted to Percellome data, i.e., absolute copy numbers of mRNA per one cell, by the homemade software SCal4 (Spike Calculation version 4). This software also graphically indicates the efficiency of in vitro transcription, the dose–response linearity of the five GSC spikes and the location of spike probe sets in the histogram of all probe sets (Fig. 1A). From the same treatment group ($n = 3$), all the pairs were plotted to a scatter graph as red (expression above detection level) or green dots (below detection level) with the data of five yellow spike probe sets (Fig. 1B). If any samples did not draw a symmetric scatter plot with yellow dot on the diagonal line, the sample were rejected for evaluation, and they were subjected to additional analyses.

Data analysis

Homemade software named RSort (Roughness Sort) [4] was used. This program sorts the probe sets as upward or downward peaks in a 3D isobologram (Fig. 2). To avoid biologically nonsense probe sets

Table 1
Summary of processed samples.

GEO accession no.	Cell types	Gene transfer	Time (h)
GSM808581	eSZ	No	0
GSM808582	eSZ	No	0
GSM808583	eSZ	No	0
GSM808584	eGP	No	0
GSM808585	eGP	No	0
GSM808586	eGP	No	0
GSM808587	eSZ	Empty vector	8
GSM808588	eSZ	Empty vector	8
GSM808589	eSZ	Empty vector	8
GSM808590	eGP	Empty vector	8
GSM808591	eGP	Empty vector	8
GSM808592	eGP	Empty vector	8
GSM808593	eSZ	EWS–FLI1	8
GSM808594	eSZ	EWS–FLI1	8
GSM808595	eSZ	EWS–FLI1	8
GSM808596	eGP	EWS–FLI1	8
GSM808597	eGP	EWS–FLI1	8
GSM808598	eGP	EWS–FLI1	8
GSM808599	eSZ	Empty vector	48
GSM808600	eSZ	Empty vector	48
GSM808601	eSZ	Empty vector	48
GSM808602	eGP	Empty vector	48
GSM808603	eGP	Empty vector	48
GSM808604	eGP	Empty vector	48
GSM808605	eSZ	EWS–FLI1	48
GSM808606	eSZ	EWS–FLI1	48
GSM808607	eSZ	EWS–FLI1	48
GSM808608	eGP	EWS–FLI1	48
GSM808609	eGP	EWS–FLI1	48
GSM808610	eGP	EWS–FLI1	48

eSZ, embryonic superficial zone; GP, growth plate.

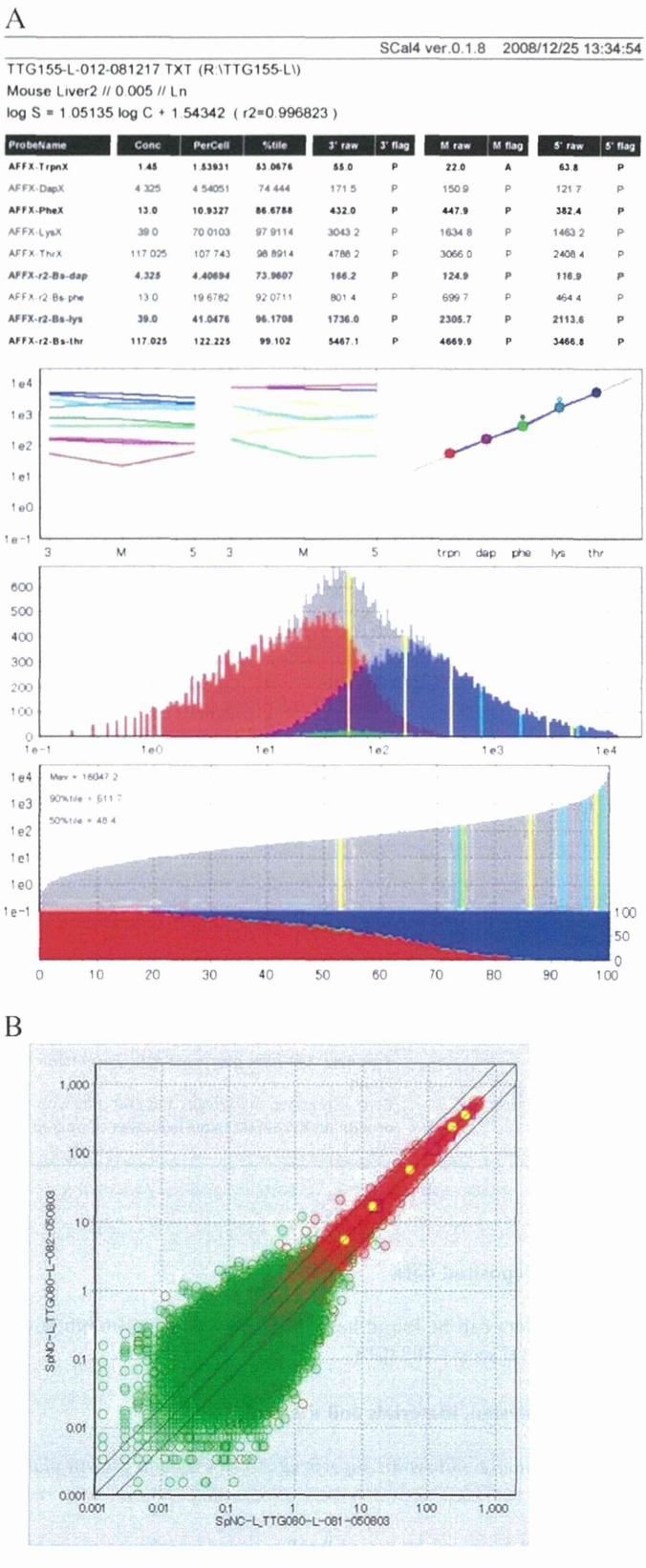

Fig. 1. Evaluation of the microarray data according to the PerCellome method. (A) An example of the SCal4 software report. SCal4 graphically indicates the efficiency of in vitro transcription, the dose–response linearity of the five GSC spikes and the location of spike probe sets in the histogram of all probe sets. (B) A scatter plot of gene expression between two experimental groups. All the pairs of probe sets were plotted to a scatter graph as red (expression above detection level) or green dots (below detection level) with the data of five yellow spike probe sets.

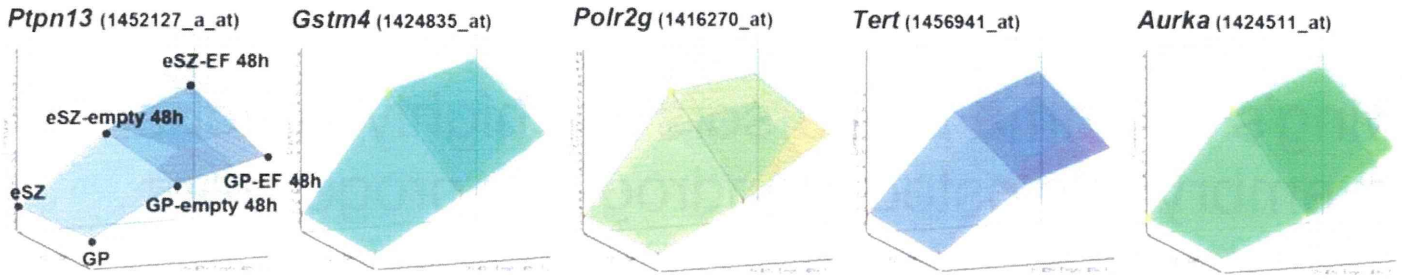

Fig. 2. Modulation of gene expression following introduction of *EWS–FLI1*. Three-dimensional grid plots of the expression of five representative genes in eSZ and eGP cells with or without *EWS–FLI1* were generated by GeneChip analysis (first two rows). The averages of each group (n = 3) were calculated and plotted as three layers of isobolograms on three-dimensional graphs as described previously [2,4].

such as ones with expression below the detection level, the data were visually checked for their 3D isobologram shape.

Discussion

We describe a unique dataset of mouse embryonic cartilage with or without the Ewing's sarcoma fusion oncogene, *EWS–FLI1*. Significantly different responses of gene expression between eSZ and eGP cells were observed. The dataset was used in the study published recently [5] and was informative to understand the tumorigenic mechanisms of Ewing's sarcoma.

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