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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

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2．研究テーマ

胚性幹細胞由来下垂体細胞の分化および細胞生物的解析

3．成果の概要（100字程度）

回転浮遊胚様体において、性腺刺激ホルモン分泌細胞の発生、分化に関連する遺伝子Hesx1、Lhx3、Prop1、GATA2、FSHβ、LHβのmRNAを発現し、またFSHβ、LHβの蛋白も発現した。蛍光免疫染色により、FSHβのみ陽性、LHβのみ陽性及びFSHβとLHβ二重陽性の細胞が見られた。一方、胚様体を腎臓被膜下に移植すると、４週間後にテラトーマの形成とその内部にFSHβとLHβ二重陽性の細胞群が認められた。

4．研 究 業 績

(1) 学会における発表　　無　・　(有)（学会名・演題）

1. 16th International Congress of the IFAA, Kyoto, August 2004: Differentiation of mouse embryonic stem cells into gonadotrope-like cells in vitro. X. Zhao, R. F. Teng, K. Asanuma, Y. Okouchi, K. Johkura, N. Ogiwara, K. Sasaki.
2. 第４回日本再生医療学会総会、大阪、2005年３月：胚性幹細胞由来の性腺刺激ホルモン分泌細胞について。趙旭、滕瑞峰、麻沼和彦、大河内康光、城倉浩平、荻原直子、佐々木克典。

(2) 発表した論文　　無　・　(有)（雑誌名・題名）

J Soc Gynecol Investig, (in press): Differentiation of mouse embryonic stem cells into gonadotrope-like cells in vitro. X. Zhao, R. Teng, K. Asanuma, Y. Okouchi, K. Johkura, N. Ogiwara, K. Sasaki.

－日中医学協会助成事業－

胚性幹細胞由来の性腺刺激ホルモン分泌細胞について

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Abstract:

This research was conducted to investigate the potential of mouse embryonic stem (ES) cells to differentiate into gonadotropes. In vitro studies used RT-PCR, Western blotting and immunohistochemistry to analyze gene expression of gonadotrope markers in developing embryoid bodies (EBs) derived from ES cells. Hesx1, Lhx3, Prop1, GATA2, follicle stimulating hormone beta (FSHβ) and luteinizing hormone beta (LHβ) mRNAs were detected at Day 6 and maintained throughout the culture to Day 56. FSHβ and LHβ proteins were expressed in EBs from Day 6 onward. Immunofluorescent labeling of FSHβ and LHβ showed that specific staining was restricted to the cytoplasm of some differentiated EB cells. With EB development, the number of positive cells increased significantly. They aggregated mainly within EBs and sparsely distributed among the outermost cells surrounding EBs. In vivo studies with implanting Day 6 EBs under the kidney capsule of 5-week-old male BALB/c nude mice exhibited clusters of cells positive for FSHβ and LHβ in the graft at 4 weeks post-transplantation. These results indicate that mouse ES cells can give rise to mature gonadotrope-like cells following their differentiation into EBs. It also shows that EBs may serve as an excellent model system to study the development and function of gonadotropes.

Key words:

Embryonic stem cell, embryoid body, gonadotrope, follicle-stimulating hormone, luteinizing hormone

Introduction:

The anterior pituitary consists of six specific hormone-producing cell types derived from a common primordium [1, 2]. Gonadotropes, one of the principle groups of cells, synthesize and secrete FSH and LH, both of which act in synergy to regulate reproduction and are structurally related glycoproteins composed of a common α subunit and a hormone-specific β subunit [3, 4]. Gonadotropin production requires the normal development and function of the pituitary gonadotropes. Many genes are involved in gonadotrope development [5]. Although abnormalities in these genes are known to result in gonadotropin deficiency, the cellular and molecular mechanisms for gonadotropin deficit remains unclear due to the lack of good model systems to study gonadotrope development and differentiation. Establishing a developmental model able to understand these mechanisms is imperative for the correct diagnosis and better treatment of reproductive disorders. ES cells are expected to become a powerful model system due to their capability of self-renewal and totipotency and the ability of EBs derived from ES cells to mimic a developing embryo[6, 7]. In this study, we investigated the potential of ES cells to undergo gonadotrope differentiation.

Materials and Methods:

Cell culture

Undifferentiated mouse ES cells (129/sv strain, Cell and Molecular Technologies, Philipsbrug, NJ, USA) were maintained on mitomycin C-inactivated STO fibroblasts in ES cell media. To induce formation of EBs after dissociation with 0.05% trypsin-EDTA (GIBCO), ES cells were suspended in differentiation media by the hanging drop method (600 cells/30 μl-drop). After 5 days (indicated as Day 6), EBs were transferred

to non-treated dishes and cultured with gentle shaking to prevent attachment for another 3-50 days.

RT-PCR

Total RNA was extracted from mouse ES cells, EBs, STO cells, pituitaries at embryonic day 12 (E12), and week 6 using RNeasy Mini Kit (QIIAGEN, Valencia, CA, USA). The RNA was reverse transcribed into cDNA using SuperScript II first-strand synthesis system with oligo (dT) (Invitrogen, Carlsbad, CA, USA). PCR was performed using Ex Taq DNA polymerase (Takara, Otsu, Shiga, Japan) in a MyCycler™ Thermal Cycler (Bio-Rad, USA).

Western blotting

EBs, mouse adult pituitary and liver were lysed on ice in lysis buffer with protease inhibitors. Proteins (10 μg) were separated by 5-15% SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The membrane was blocked in phosphate-buffered saline (PBS, PH 7.4) containing 3% serum (Funacoshi, Tokyo, Japan) of the same species as secondary antibodies, and then probed with rabbit anti-mouse FSHβ polyclonal immunoglobulin (Ig) (1:3000, Biogenesis, Kingston, NH, USA) or goat anti-rat LHβ polyclonal IgG (1:1000, Santa Cruz Biotechnology, California, USA). Primary antibodies were visualized by the addition of horseradish peroxidase (HRP) goat anti-rabbit IgG (1:1000) or rabbit anti-goat IgG (1:500, Bio-Rad, Hercules, CA, USA) and developed by 3, 3' diaminobenzidine tetrahydrochloride (DAB, Wako, Tokyo, Japan).

Transplantation of EBs under kidney capsule

20 EBs were harvested from hanging drop cultures after 5 days and then transplanted under the kidney capsule of 5-week-old male BALB/c nude mice (Charles River Japan, Yokohama, Japan). The recipients were examined for donor EB survival and differentiation in vivo using immunofluorescence at 4 weeks post-transplantation. All animal experiments were conducted in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

Immunocytochemistry

Single immunofluorescent label of gonadotropes

EBs and control mouse pituitary were routinely fixed in 4% paraformaldehyde and embedded in paraffin. After deparaffinization and rehydration, 5-μm thick sections were microwaved in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval, blocked with 3% host serum of secondary antibodies in PBS (pH 7.4) to reduce non-specific binding, then incubated with rabbit anti-mouse FSHβ polyclonal Ig (1:2000) or goat anti-rat LHβ polyclonal IgG (1:100). Primary antibodies were detected using Alexa fluor 488 conjugated with goat anti-rabbit IgG or donkey anti-goat IgG (1:200, Molecular Probes, Eugene OR, USA).

Double label of gonadotropes

In the colocalization experiment of FSHβ and LHβ, first, paraffin-embedded sections were reacted with goat anti-rat LHβ polyclonal IgG (1:100) and detected using donkey anti-goat IgG conjugated with Alexa fluor 488 (1:200). Then, they were subjected to rabbit anti-mouse FSHβ polyclonal Ig (1: 2000) and visualized by goat anti-rabbit IgG conjugated with Alexa fluor 568 (1:200, Molecular Probes, Eugene OR, USA). Specimens were examined using a confocal laser scanning microscope.

Results:

EB differentiation was recorded by phase-contrast microscope (Fig1). EBs in A, B, C and D were harvested at Day 6, 9, 12, and 56 respectively. With the prolongation of EB culture, there was an obvious increase in EB size due to cell multiplication, although the degree of multiplication among EBs varied. The period of EBs' expansion continued for 12-14 days after they were collected from hanging drops and the volume of EBs remained unchanged thereafter. The out layer of EB was the visceral endoderm indicated as arrows.

RT-PCR analyses

β-Actin was employed as the endogenous control and detected in all the samples. Fetal pituitary was used as the positive control for Hesx1, Lhx3 and Prop1, while adult pituitary provided positive control for GATA2, FSHβ, LHβ and Pit1. Undifferentiated ES cells expressed only Hesx1, but EBs from Day 6 onward expressed all of these gene transcripts except Pit1. No bands were exhibited in STO cells [Fig. 2].

Western blot analyses

Both FSHβ (mass 14919) and LHβ (mass 15028) proteins were detected in western blots of all EB samples and adult pituitary, but not in liver (Fig 3).

Immunocytochemical analyses

Few cells were found to be positive for both FSHβ and LHβ on Days 6, 9, 15, and 21. Only a small number of FSHβ (Fig. 4A) or LHβ (Fig. 4B) positive cells were detected at this stage. Cells labeled for FSHβ only (Fig. 4C) or LHβ only (Fig. 4D) significantly increased in Day 35 and 56 EBs. Cells double-stained for FSHβ and LHβ similarly increased during the same period (Fig. 4E and F). These positive cells were clustered together within the EBs or were sparsely scattered in the outermost cells surrounding them. In control pituitary, positive staining was only observed in those specific cells of the anterior lobe, but not in the posterior (neural) lobe at all (Fig. 4G). Control EBs failed to show specific labeling when primary antibodies were omitted (Fig. 4H). Teratoma formed and exhibited clusters of cells positive for FSHβ and LHβ (Fig. 5) at 4 weeks post-transplantation.

Conclusion:

These results indicate that mouse ES cells can give rise to mature gonadotrope-like cells in EBs. It also shows that EBs may serve as a novel model system to study the development and function of gonadotropes.

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注：本研究は、2002年8月26日『6th International Congress of the IFAA』と2003年3月2『第4回日本再生医療学会総会』にてポスター発表、in vitroの実験に関しては『J Soc Gynecol Investig』誌に掲載予定。

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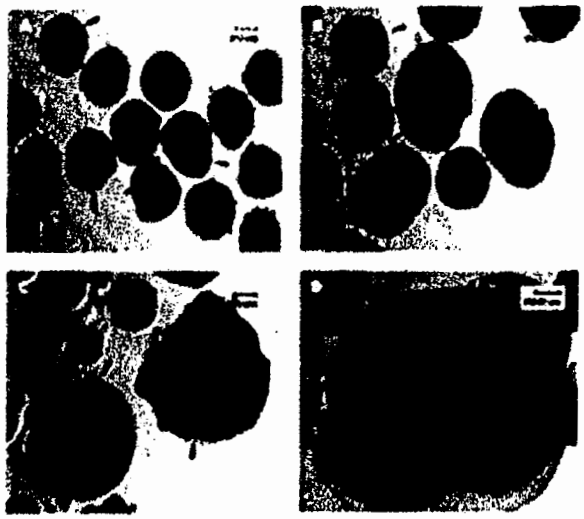
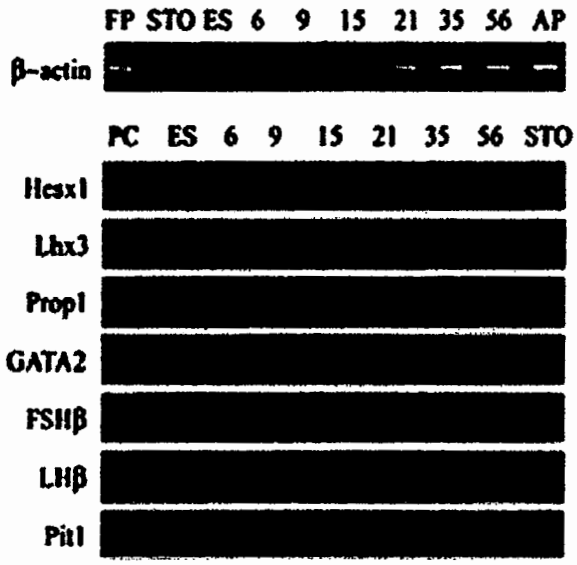


Figure 1



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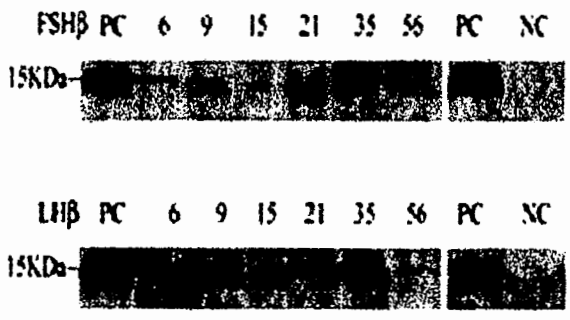

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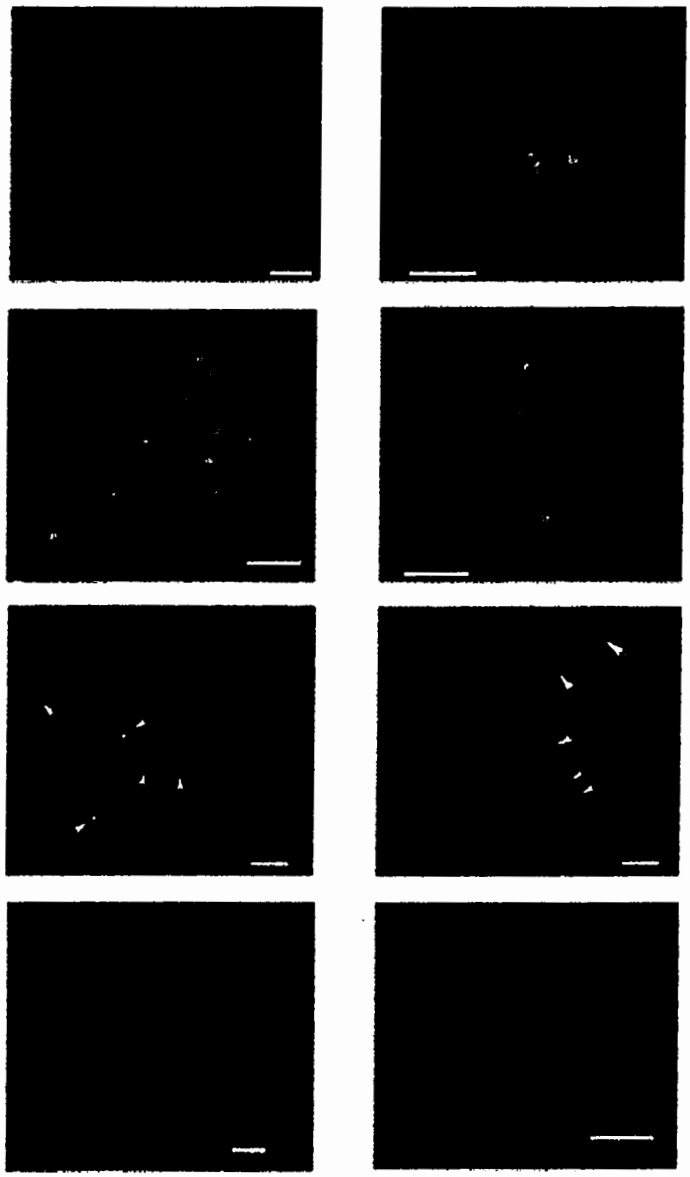


Figure 4

Figure 5

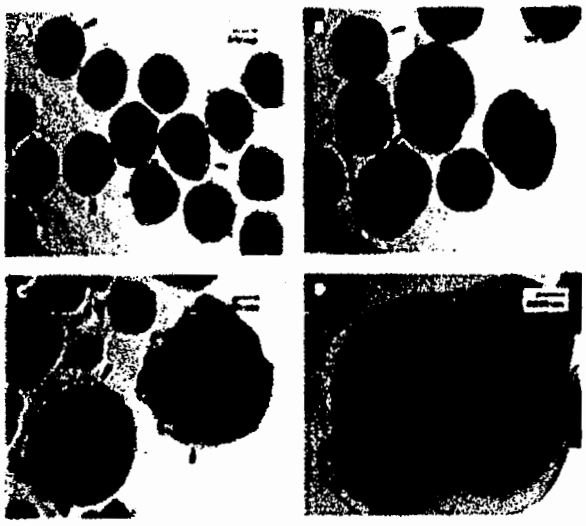
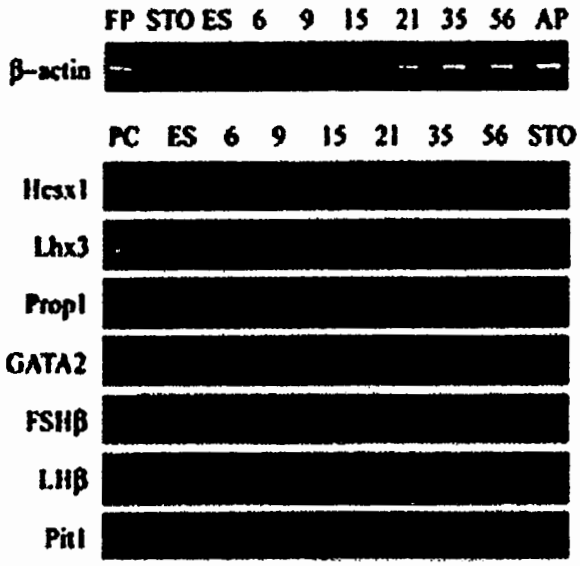


Figure 1



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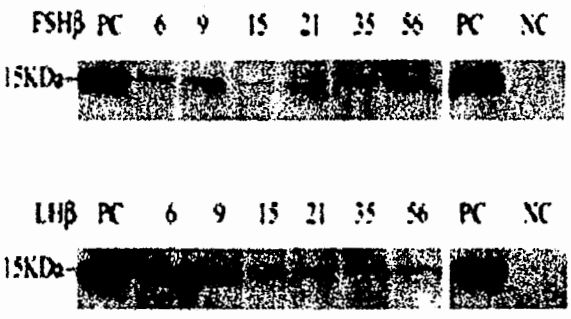

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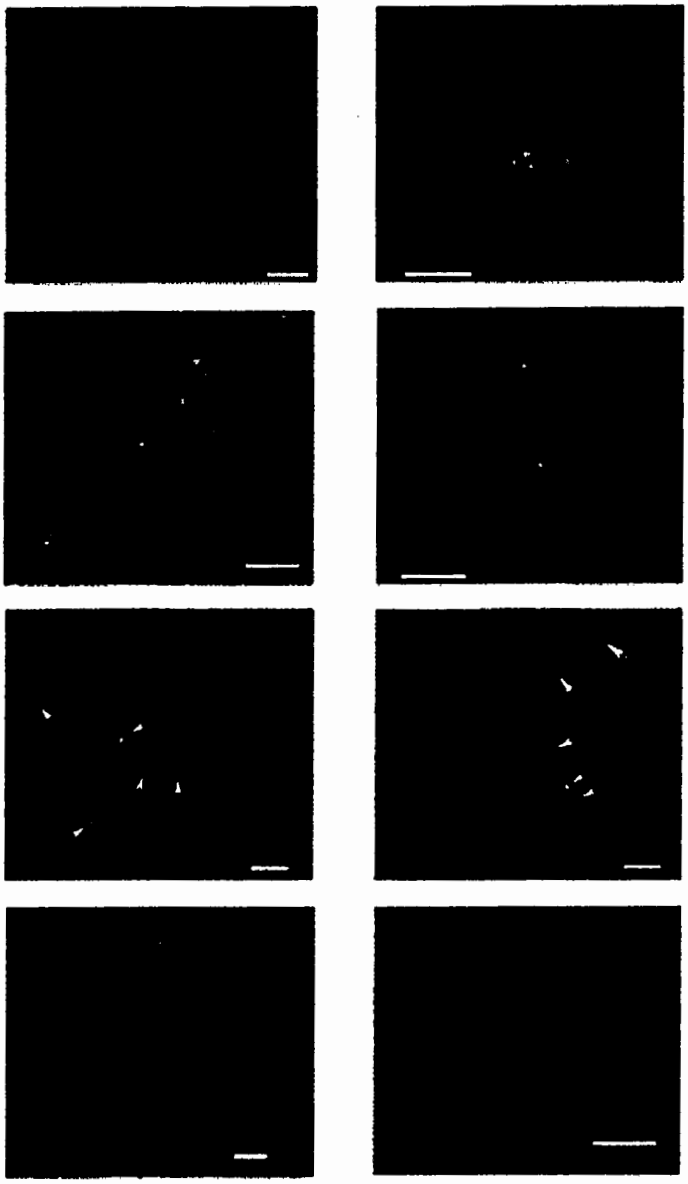

Figure 4

Figure 5