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学位の種類	博士（医学）
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学位論文題目	Establishment of a culture model for mouse neonatal ovary （マウス新生児卵巣培養系の確立）
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別紙様式３（課程博士・論文博士共用）

論　文　内　容　要　旨

※整理番号	786	氏名（ふりがな）	鄭　璐藝（郑　璐艺）（てい　ろげい）
学位論文題目	Establishment of a culture system for mouse neonatal ovary （マウス新生児卵巣培養系の確立）		

Background:

Oocytes, female germ cells involved in reproduction, are stored as dormant primordial follicles in ovaries. Each primordial follicle contains an immature oocyte arrested at the diplotene stage of meiosis and is surrounded by a layer of supporting flattered somatic forming a spheroid aggregation in the ovary. The activation of primordial follicles is the initial step in the complex process of oocyte growth, and is accompanied by the proliferation and differentiation of the surrounding pregranulosa cells and followed by the ovulation. The molecular mechanism underlying the activation of primordial follicles is not known well. At birth women have a million of follicles and they are not renewed during live. The number of primordial follicles remaining in the late 30s is estimated to be approximately 2-5% of those present at birth.

Infertility of women is an issue in developed countries. where many women desire having a child later in life. Infertility related to aging is due to diminution of the oocyte pool. Thus, the development of methods to prevent age-related oocyte depletion may preserve women fertility and improve pregnancy rate of infertility treatment for older women in reproductive age.

In vitro culture system of mouse neonatal ovary was established as a model to study the pharmacological effect of drug on the survival of oocytes and their supporting cells, and assess their effect on the cell signaling pathways.

Objective:

The direct effect of culture in vitro on the survival of oocytes and supporting cells has not been studied in detail although *in vitro* culture systems of rodent neonatal ovary have been used for the study of primordial follicle activation. The aim of the present research is to study the direct effects of *in vitro* culture on the survival of oocytes and their supporting cells in the mouse neonatal ovary.

（備考）１．論文内容要旨は、研究の目的・方法・結果・考察・結論の順に記載し、２千字
程度でタイプ等を用いて印字すること。
２．※印の欄には記入しないこと。

別紙様式３の２（課程博士・論文博士共用）

（続紙）

Methods :

The morphology of the ovary and the proliferation and cell death of primordial and primary follicle were compared between the 7-day-old mouse ovary and the mouse ovary obtained at post-neonatal 3 day and subsequently cultured for 4 days (cultured ovary).

1. Ethics and animal care: All C57/BL6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan), were maintained on 12-h light, 12-h dark cycle and given food and water ad libitum.

2. Neonatal ovary collection, culture and morphological analysis: Female mice were sacrificed and their ovaries were removed at post-neonatal 3 or 7 day. Ovaries of 3-day-old mice were cultured for 4 days at 37 ℃ and culture medium was changed every two days. After 4 day culture, ovaries were fixed in Bouin's solution, embedded in paraffin by routine histological procedure, and sectioned at 4 μm. Six sections in the edge of the ovary and three sections in the middle of the ovary were stained with hematoxylin and eosin, then the ovarian structure was analyzed and the numbers of primordial and primary follicles were counted. Seven-day-old mice ovaries were collected, fixed in Bouin's solution, proceeded and evaluated with the similar method for the cultured ovaries.

3. Staining for Ki67 and cleaved poly(ADP-ribose) polymerase 1(PARP-1): The sections adjacent to those used for morphological analysis were used for immunohistochemistry of KI67 and for immunofluorescence of cleaved PARP-1 to analyze proliferation and cell death, respectively. The analysis of proliferation in granulosa cells (GCs): The sections were subjected to antigen retrieval (10 mM citrate buffer; pH 6.4), treated with 3% hydrogen peroxide, incubated in mouse antihuman Ki-67 (abcam, 1:2000), incubated in horseradish peroxidase-conjugated secondary antibody, followed by DAB, counterstained with hematoxylin and mounted with cytoseal. The numbers of GC or pregranulosa cell (pGC) stained with Ki67 in primary or primordial follicles were counted, and the proportion of the numbers of GC or pGC stained with Ki67 per total numbers of GCs or pGC in primary and primordial follicles was calculated. The analysis of cell death in oocytes: The slides were subjected to antigen retrieval (10 mM citrate buffer; pH 6.4), treated with 3% triton X-100, incubated in mouse antihuman cleaved-PARP-1 (Cell Signaling Technology; 1:100), incubated with Alexa Fluor 568 goat antibody, treated with DAPI and mounted in Fluorescent Mounting Media. After obtaining pictures by fluorescent microscopy, the slides were soaked in water and the cover slips detached. Slides were stained again with hematoxylin to evaluate the ovaries structure by light microscopy. The numbers of oocyte stained by cleaved PARP-1 in primary and primordial follicles were counted and the ratio of Ki67-stained oocyte per total number of oocyte was calculated. Statistical analysis: All data points represent the mean of measurements and are expressed as the mean ± standard error of mean. GraphPad Prism ver.7 was used to perform Student's t-test, with a significance threshold of $p < 0.05$.

Results:

There was an area consisting of a large number of stromal cells and piknosis in the middle of the cultured ovary whereas a number of secondary follicles exist in the middle of 7-day-old ovary.

In the edge of ovary, the 1st to 16th sections from the edge, there was no difference between the ovary of 7-day-old mouse and the cultured ovary with regard to the morphology of primordial follicles and primary follicles and the primordial follicle activation to primary follicle. The proliferation of granulosa cells in the primary follicle and pregranulosa cells in the primordial follicle was not different in the edge of ovary whereas the proliferation of granulosa cells in the primary follicle was less in the middle of cultured ovary compared with the ovary of 7-day-old. More number of oocytes in primary follicles tend to go into apoptosis in the middle of the cultured ovary.

Based on these results, sections 16 or less from the edge of the ovary obtained at post-neonatal 3 day and subsequently cultured for 4 days are adapted to the research of primordial follicle activation when the effect of a medicine such as synthetic hormone on the primordial follicle activation is studied.

Discussion:

We concluded that the sections in the edge of ovary, sections 16 or less from the edge, were given less detrimental effect by culture with regard to the activation of primordial follicle to primary follicle.

A large number of targeted gene in mice has been used to study the signaling related to primordial follicle activation. This method is superior to culture experiment of neonatal ovary regarding the research for the nature and biology of primordial follicle activation. However, the culture experiment is more suitable when the direct effect of a medicine such as synthetic hormones on the primordial activation and its mechanism is researched.

Conclusion: In conclusion, we established a neonatal ovarian culture system and assessed its efficacy and limitation for evaluation of primordial follicle activation. Using this model the effect of a synthetic substance can be evaluated.

別紙様式８（課程・論文博士共用）

学位論文審査の結果の要旨

整理番号	786	氏　名	鄭　璐藝
論文審査委員			

（学位論文審査の結果の要旨）（明朝体１１ポイント、６００字以内で作成のこと。）

原始卵胞は、出生時に最も多く、成長と共に激減する。原始卵胞の活性化の分子メカニズムを明らかにする目的で、新生仔マウスの卵巣を用いて卵細胞の発生を評価する試験管内アッセイ系を確立することを試みた。

生後３日目のマウスから卵巣を摘出し、試験管内で４日培養した。対照として、生後７日齢の新生仔卵巣を用いた。両卵巣を固定後に４ミクロン厚の切片作成し、両者を比較検討した。その結果、培養卵巣の中心部では細胞死が認められたが、それ以外では原子卵胞や一次卵胞に形態学的な差異はなかった。一次卵胞数/原始卵胞数、死細胞数にも差異は認められず、正常な発生が認められ、評価系として成立することを見出した。

学位論文に関連して、本評価系を用いてジエノゲストの効果を検討してところ、ジエノゲストが卵巣中の卵胞を原始卵胞の状態で停止させ、産子数の増加させることを示した。

以上より、本論文は新生仔マウスを用いた卵細胞の発生を評価する新しい試験管内アッセイ系を確立し、その有用性を示したものである。さらに、最終試験として論文内容に関連した試問を受け合格したので、博士（医学）の学位論文に値するものと認められた。

（総字数　488 字）

（平成２９年　１月２４日）