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Acknowledgements to individuals and to funding sources are appropriate in this section.

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Contents

Heterogeneity of pluripotent stem cells shows a basic manner of self-renewing stem cells

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Abstract

Pluripotent stem cells that can indefinitely propagate *in vitro* are derived from groups of pluripotent cells in pre- and post-implantation embryos. Mouse embryonic stem (mES) cells and post-implantation epiblast-derived ES (mEpiS) cells, both pluripotent stem cells, are derived from the inner cell mass (ICM) of blastocyst and the epiblast of post-implantation embryo, respectively. Although these pluripotent stem cells are thought to be a homogenous cell population, recent studies reveal that there is apparent heterogeneity. This article discusses the significance of the heterogeneity observed in pluripotent stem cells, based on current findings including our studies. Of particular interest, our study detected heterogeneous expression of *Stella*, a marker of ICM and primordial germ cells (PGCs), in both mES cells and EpiS cells. Intensive analyses of heterogeneous *Stella* expression illustrate the nature of pluripotent stem cells.

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Heterogeneity of mES cells

mES cells are pluripotent cells originated from the inner cell mass (ICM) of blastocysts. However, unlike the cells *in vivo*, mES cells retain pluripotency and exhibit the capacity for indefinite self-renewal, while the cells *in vivo* undergo differentiation according to a strict developmental program. As long as mES cells are cultured in an appropriate medium, they can undergo self-renewal without compromising pluripotency. For this reason, mES cells are generally regarded as a homogeneous group of cells in the majority of studies. Recent studies reveal, however, that a number of genes, such as *Stella* (or *Dppa3*), *Zfp42*, *Pecam1* and *Nanog*, are heterogeneously expressed in mES cells, apparently showing that mES cells are composed of heterogeneous cell populations. Of these genes heterogeneously expressed in mES cells, we have focused on the nature of heterogeneous expression of *Stella*, a definitive marker of the germ cell lineage. *Stella* expression is first observed in preimplantation embryos, thereafter repressed in the epiblast (Payer et al., 2006; Sato et al., 2002), and subsequently re-expressed only following specification of PGCs (Payer et al., 2006). Based on analysis using Stella: GFP reporter mES cells in which GFP expression is driven by the *Stella* promoter, only 20-30% of the mES cells exhibit Stella-GFP expression (Hayashi et al., 2008). Interestingly, under culture condition supporting self-renewal of mES cells, each sub-population, Stella-GFP-positive and Stella-GFP-negative population, were mutually interchangeable; the purified Stella-GFP-positive (or –negative) population could reconstitute the parental proportion of the heterogeneity. This result is consistent with other studies showing that any of purified subpopulation, for example Nanog-negative mES cell population, can reconstitute the parental proportion of the heterogeneity (Chambers et al., 2007; Furusawa et al., 2004; Toyooka et al., 2008). These clearly demonstrate that mES cells are not a homogeneous cell group but rather exhibit meta-stability on which the cells fluctuate between at least two states.

Gene expression analysis using the subpopulations of Stella-GFP mES cells demonstrated that *Stella*-positive mES cells are closely

related to the ICM, whereas *Stella*-negative cells are more related to the epiblast (Hayashi et al., 2008). Consistent with this observation, expression of other genes showing heterogeneous expression in mES cells, such as *Zfp42*, *Pecam1* and *Nanog* is largely correlated; for example Pecam1-positive cells are enriched in *Nanog* transcripts (Furusawa et al., 2006). Strikingly, all these genes are expressed in the ICM, but not in the epiblast. Combined with the observed meta-stability of mES cells, it is feasible that mES cells in the undifferentiated state fluctuate between an ICM-like and epiblast-like status (Figure 1). It is not the case that epiblast-like mES cells are simply emerging differentiated cells in culture, as isolated epiblast-like cells revert to ICM-like cells
restoring the balance. As described above, mES cells are cells indefinitely

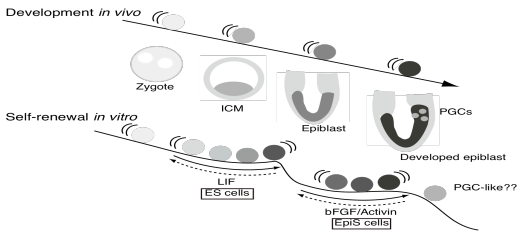

Figure 1. A basic manner of self-renewal of mouse pluripotent stem cells *in vitro* and comparison to their counterparts *in vivo*. After fertilization, cell differentiation irreversibly proceed according to a strict program for development (Development *in vivo*). In contrast, pluripotent stem cells, ES cells and EpiS cells derived from the ICM and the epiblast respectively, can arrest the developmental program under an appropriate condition. To maintain the pluripotent cell population, they fluctuate between subpopulations reflecting the *in vivo* counterpart (Self-renewal *in vitro*).

self-renewing *in vitro*, whereas their origin, the ICM, undergo differentiation according to a defined developmental program. Thus, it is possible that mES cells to some extent mimic the natural differentiation process *in vivo*, leading to the down-regulation of ICM-specific genes and the up-regulation of epiblast-specific genes, and thereafter mES cells revert from the epiblast-like status to the ICM-like status by still unknown mechanism(s). The latter process may in fact be a *bona fide* reprogramming process. Supporting this idea, recent studies demonstrates that mES cells can be derived not only from the ICM of blastocysts at embryonic day (E) 3.5, but also from epiblast cells from later developmental stages, even from E7.5 epiblast, under appropriate culture conditions in the presence of Leukemia Inhibitory Factor (LIF), an important cytokine for mES cell maintenance (Bao et al., 2009). These observations suggest that the appropriate culture conditions evoke a reversion of the process of differentiation. This is quite important to understand the mechanisms underlying the reversion process that illustrates the nature of self-renewal of mES cells.

Considering the intrinsic heterogeneity of mES cells may also be paramount to achieve direct differentiation of mES cells into specific cell lineages, as this heterogeneity may reflect functional differences within the population of mES cells. For instance, our study demonstrated that Stella-positive and Stella-negative cells exhibit distinct differentiation potential. When individual cell populations were cultured under condition promoting trophectoderm differentiation, Cdx2-positive trophectoderm cells emerged from only Stella-negative cells (Hayashi et al., 2008). Although it is generally known that mES cells are not prone to differentiate into trophectoderm cells, our study reveals that such trophectoderm-potent cells are enriched in the Stella-negative population. This may partially explain why it is virtually impossible to induce homogenous differentiation in mES cells, as reactivity to differentiation-inducing factor(s) seems to vary in each sub-population. It is also noteworthy that some, but not all, Stella-negative cells differentiate into trophectoderm cells, indicating that Stella-negative cells can be distinguished into even smaller sub-populations. Making mES cells homogenous is therefore a prerequisite

for directed mES cell differentiation.

Heterogeneity of epiblast stem cells

As described above, mES cells fluctuate between an ICM-like and epiblast-like state under culture condition with LIF. In contrast, recent reports demonstrate that mES cells completely convert into epiblast-like state by cultivation with basic fibroblast growth factor (bFGF) and Activin A, instead of LIF. The converted mES cells indefinitely propagate under these conditions, while keeping pluripotency and features of epiblast cells (Guo et al., 2009). The new pluripotent cells are named epiblast stem (EpiS) cells. EpiS cells are originally derived from E5.5-6.5 epiblast cells and it has been proven, as described above, that EpiS cells can also be derived from mES cells under the appropriate culture conditions (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007). Under those conditions, the derived EpiS cells no longer contain ICM-like mES cells. EpiS cells express epiblast marker genes and interestingly show epigenetic features similar to those observed in the epiblast. Of those common epigenetic features, it is noteworthy that one of the two X chromosomes is inactivated in female (XX) EpiS cells. In female somatic cells, one of the two X chromosomes is transcriptionally silenced, so that dosage of X-linked gene transcripts is equivalent to that in male cells. However, the germ cell lineage, including pluripotent cells, is the exception. In the case of female mice, both X chromosomes are active in primordial germ cells, the ICM and mES cells. It is suggested that pluripotent cell-specific transcription factors, such as Oct4, Nanog and Sox2, play a role in keeping both X chromosomes active in mES cells and ICM (Navarro et al., 2008). However, despite expression of all the factors in EpiS cells, one of the X chromosomes remains inactive. Thus, it is of particular interest to study how EpiS cells prevent the silent X chromosome from being reactivated.

Given that the fluctuation between sub-populations, reflecting the *in vivo* counterpart, is a common feature of pluripotent stem cells, EpiS cells would be composed of a heterogeneous population that would reflect E5.5-6.5 epiblast. This is the case, as we found at least heterogeneous expression of

Blimp1 in EpiS cells. Blimp1 (or Prdm1) is known as a transcriptional repressor involved in germ cell specification and is the earliest marker for primordial germ cell (PGC) precursors that emerge in the posterior-proximal part of the E6.25 epiblast (Hayashi and Surani, 2009). Using Blimp1-GFP reporter EpiS cell lines, we detected Blimp1-GFP expression in a significant proportion (10 to 50%) of the self-renewing Blimp1-GFP EpiS cells. Correlated with the expression of Blimp1, an early PGC marker gene, transcripts of additional early germ cell marker genes, such as *Prdm14* and *Nanos3*, were selectively enriched in Blimp1-positive subpopulation of the EpiS cells. This finding suggests that a certain subpopulation of EpiS cells undergoes differentiation into *bona fide* PGCs. Indeed, we detected expression of *Stella*, a marker for definitive PGCs, in part of the Blimp1-positive population, though the proportion of Stella-positive cells was rather low (0-2%). Using Stella-GFP reporter EpiS cells, we characterized the population of Stella-positive putative PGCs present in EpiS cells. The putative PGCs capture features of PGCs *in vivo*, as they give rise to EG cells, undergo epigenetic reprogramming and enter meiosis. Interestingly, we also found that in contrast to PGC specification *in vivo* where fate of Blimp1-expressing epiblast cells is basically restricted to PGCs, a part of Blimp1-positive EpiS population was able to give rise to Blimp1-negative EpiS cells and reconstitute the parental proportion. This means not only that similar to mES cells, EpiS cells also fluctuate, while keeping pluripotency (Figure 1); but also that, in aggrement with our previous findings, Blimp1-positive cells are not lineage restricted yet but still need inductive signals to become *bona fide* PGCs (de Sousa Lopes et al., 2007). Although it remains unclear whether other type of subpopulations exist in EpiS cells, it is feasible that EpiS cells fluctuate between several subpopulations corresponding, for example, to anterior and posterior specific lineage progenitors present in the epiblast. This possibility can be evaluated by using anterior or/and posterior epiblast-specific gene reporter EpiS cells.

Human embryonic stem (hES) cells are thought to be close to mouse EpiS cells, rather than to mES cells, as they exhibit similarities with respect to morphology, cytokine requirements and

differentiation potential. Thus, EpiS cells might prove to be a good model to study pluripotency in humans. Consistent with our observation that PGC are spontaneously differentiated from EpiS cells, a recent report has showed that hES cells also generate continuously PGC lineage while keeping pluripotency (Clark et al., 2004; Clark and Reijo Pera., 2006; Bucay et al., 2009). Considering that heterogeneity is commonly observed in pluripotent/multipotent stem cells *in vitro*, it is feasible that hES cells are also composed of heterogeneous cell populations. Using mES and EpiS cells, we propose that each subpopulation has distinct differentiation capacities. Then, in the case of hES cells, it might also be important to control the heterogeneity for directed and homogenous differentiation into a specific cell lineage.

Control of heterogeneity

It has remained unclear how to control heterogeneity. However, it is apparent that the culture condition has a significant impact on the degree of heterogeneity. For example, our study revealed that the proportion of Stella-positive mES cell population increased, when cultured on mouse embryonic fibroblasts (MEFs), suggesting that MEFs push mES cells into a more ICM-like status. On the other hand, culture in chemically defined medium decreased the size of the Stella-positive cell population. Detail analysis suggested that the chemically defined medium placed mES cells at an intermediate position between ICM- and epiblast-like cell populations (in preparation). We are now testing whether these relatively homogenous cell populations exhibit homogenous differentiation into specific cell lineages. As described here, heterogeneity of mES cells show a basic manner of self-renewing pluripotent stem cells *in vivo*. Perhaps, self-renewal, defined as cell division generating two completely identical daughter cells, might not exist in pluripotent stem cells *in vitro*. Further studies are required to fully understand the mechanisms underlying the heterogeneity and its role in pluripotency.

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2009 年

第 11 回 SSRE シンポジウム

講演要旨集

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生殖幹細胞の分化制御機構

2009 年 3 月 7 日(土曜日)

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SSRE 生殖工学研究会

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精巣幹細胞から前駆細胞への分化に伴う遺伝子発現制御機構の変化

○大保　和之（横浜市立大学医学部組織学）

近年、再生医学の実現に向けて、ES 細胞、iPS 細胞に代表される多能性幹細胞研究に加え、組織幹細胞研究も急速な進展をみせている。幹細胞研究の視点に立てば、組織にある細胞は、幹細胞（stem cell）、前駆細胞（progenitor cell）、分化細胞（Maturing cell）の大きく3つに分類する事ができる。組織幹細胞は多能性幹細胞と異なり、すでにある特定の細胞系譜に運命決定づけられてはいるが、自己複製能、増殖能、分化能、組織再構築能を維持している細胞であり、前駆細胞は、このうち自己複製能を喪失した細胞である。幹細胞、前駆細胞の性状を研究する理由は、生物学的興味ばかりでなく、*in vitro* における幹細胞増幅が可能となれば、組織再生の実現化は容易となるなど、その成果が社会に大きく還元されるからであると考えられる。

生体内において組織幹細胞を持つ組織として精巣は古くから知られており、形態学的手法による細胞の分化段階の分類が精力的になされてきた。しかし、造血幹細胞の研究が最も進んでいることからも解るように、純化した特定の細胞集団を材料に、分化能、自己複製能の評価を行うシステムの構築が幹細胞研究には必要不可欠であり、形態学的解析には限界がある。そこで我々も含め幾つかのグループが、精巣において、幹細胞、前駆細胞に特異的に発現する細胞表面分子、或は、特異分子の発現調節領域を用いて GFP を発現させたような遺伝子改変マウスを作出、利用し、幹細胞、前駆細胞を生細胞のまま純化単離することを試み、さらに、純化されてきた幹細胞候補細胞の精細管移植による精巣再構築能の検証を行い、より厳密に、幹細胞、前駆細胞の性状解析を行っている。我々は転写因子 Oct4 が生殖細胞系譜に特異的に発現していることから、その発現調節領域を用いて GFP 蛋白を発現するレポーターマウスを作出し、c-Kit の発現の有無により、幹細胞、前駆細胞の区別が可能であることを明らかにした。現在は、これに加え、他のグループから c-Kit 陰性の精原細胞に特異性が高いNeurogenin 3, Plzf, 我々のグループからJAM4などといった新たなマーカーが明らかとなってきている。

このようなマーカーの同定とマーカーとしての信頼性の検証は、幹細胞研究に必要不可欠な研究であり継続して行っているが、その一方でマーカーの発現は、細胞周期、マウスの系統、遺伝子改変の影響により時に定常状態とは異なる表現型とな

ることが知られている。そこで我々は、現在より普遍的な幹細胞、前駆細胞の鑑別が可能か、高次の遺伝子発現制御機構（エピジェネティクス）の視点から詳細に検討を行ってきた。定常状態のマウスを用い、上述した幹細胞、前駆細胞のマーカーに重ね合わせて DNA メチル基転移酵素、いくつかのヒストン修飾酵素の免疫染色パターンを観察した。その結果、より未分化な幹細胞分画では、de novo DNA メチル基転移酵素の発現がなく、ゲノムは低メチル化状態になっており、分化細胞には存在する抑制性ヒストン修飾の一部が認められなかった。前駆細胞への分化の進行とともに、de novo DNA メチル基転移酵素の発現と、幹細胞では認めなかった抑制性ヒストン修飾が加わってくることが判った。また、幹細胞、前駆細胞で発現が認められるいくつかの遺伝子を代表例としてピックアップし、その発現調節領域を中心に、ゲノムメチル化、ヒストン修飾状況を観察すると、例えば前駆細胞で必須な分子 c-Kit の発現調節領域は、発現が認められない幹細胞の時期でも発現調節領域における DNA のメチル化という制御機構は用いられておらず、前述した免疫組織学的解析の結果を反映していたことに加え、幹細胞における遺伝子発現抑制機構は、あるヒストン修飾による抑制機構が主であった。

現在、組織幹細胞研究は、特異的マーカー候補分子の同定、マーカーとしての信頼性の検証の繰り返しを行うなかで、幹細胞集団が、いわゆる actual stem cell と potential stem cell の2つに区別されはじめるなど、分子論的に新たな分類が行われ始めている。我々は、これらの進展に重ね合わせて幹細胞、前駆細胞のゲノム修飾機構を観察しているが、将来の検討課題の1つとして、その成果が他の組織幹細胞システムに応用可能なものであるか検証が必要と考えている。

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生殖細胞分化のエピジェネティカル制御

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多細胞有性生殖生物では、体の構築や機能に影響を持たない特別な細胞である配偶子が、次世代を生み出し、遺伝情報を継承する。従って、配偶子はもちろん、そのもととなる生殖系列細胞も体細胞には無い多くの特性を持つ。基本的に細胞の特性は、利用する遺伝子の組み合わせによって生み出される。生殖細胞は、持っているゲノム情報は体細胞と同じであるが、特異的な遺伝子を多く発現するための特有の制御システムを持つ。一般的に遺伝子発現制御は、プロモーターを中心としたシス配列とトランスに作用する転写因子との組み合わせによる直接的な制御と、クロマチン構造変化を伴うエピジェネティカル動態による間接的な制御に大別される。生殖系列細胞は多くの特異的な基本転写因子群と、転写制御因子群を持ち、さらにゲノム全体のエピジェネティカル変動が著しいことが知られている。しかしそれらが、生殖細胞の特徴的な遺伝子制御と具体的にどのようにつながるのかについては、実はよくわかっていない。本講演では、マウス精巣生殖細胞分化における特異的な遺伝子制御システムの理解を目的とした研究成果について紹介する。

プロモーター制御

トランスジェニックマウス解析と in vivo electroporation 法の併用により、精細胞特異的遺伝子の発現制御領域の同定を試みた。その結果、TATA-box 等の既知のプロモーターエレメントを持たないが cAMP response element (CRE)を含む 、転写開始点前後の非常に短い配列だけで特異的発現制御に必要十分であることを見いだした。さらに CRE への転写因子 CREM の結合が、基本転写活性に必要な例を見いだした。CREM は精細胞の分化に必須であることが示されていることから、基本転写因子群と CREM を含む転写制御因子が近接して作用するコンパクトな転写制御メカニズムが生殖細胞に特有のシステムの確立に役立っている可能性がある。また、精巣生殖細胞だけで発現する遺伝子にはレトロポゾンと思われる単一エクソン遺伝子が比較的多く含まれる。レトロポゾンは親遺伝子由来の mRNA が逆転写活性により cDNA となった後、ゲノム中にランダムに挿入されるため、プロモーターを持たず発現されないので、ほとんどは偽遺伝子となる。一方、上述の通り、精巣生殖細胞は典型的なプロモーターではない配列からも転写しやすい制御システムを持つので、一部のレトロポゾンの発現が

可能となり、それらが生殖細胞だけで発現する機能的遺伝子として定着した可能性が示された。

エピジェネティカル制御

哺乳動物ゲノムでは、遺伝子プロモーター上流のメチルCpG密度が高い場合、発現が抑制されることが知られている。特にCpG頻度の高いCpGアイランドは通常どんな細胞でもメチル化されない。ところが最近、体細胞組織でCpGアイランドがメチル化されるいくつかの遺伝子が同定されたが、それらは雄生殖細胞ではメチル化されない。一方、先に述べた通り、精細胞では多くのイントロンレス遺伝子が特異的に発現しており、これらは遺伝子上流ではなく、内部のCpG頻度が比較的高い。 そこで、イントロンレス遺伝子の転写開始点近傍から下流にかけての CpG メチル化パターンと発現との相関を体細胞と生殖系列細胞で調べた。その結果、イントロンレス遺伝子の半数以上は体細胞ではメチル化によって発現が抑制され、生殖細胞での発現には脱メチル化が必要条件であることがわかった。ただし、生殖系列を通じて低メチル化であることと、CpG 密度が高い一部の遺伝子は体細胞でもメチル化されていないことから、メチル化消去が転写開始と直接関連することは無く、別の抑制機構の存在とその解除が必要であろうと考えた。そこでヒストン修飾について調べたところ、精細胞における発現と転写活性型ヒストンメチル化修飾との相関は見られたが、精母細胞以前の発現抑制と、転写抑制型ヒストンメチル化修飾の相関については今のところ観察されていない。一方、染色体レベルのヒストンメチル化修飾の変動は全般的な遺伝子発現と相関しているように見える。

これら個々の遺伝子制御における DNA メチル化とヒストンメチル化の役割とゲノム全体の変動との関連を含めて、生殖細胞分化のエピジェネティカル制御について議論したい。

胚性幹細胞の自己増殖様式と始原生殖細胞分化

○林克彦（The Gurdon Institute, University of Cambridge）

哺乳類（主にマウスおよびヒト）の胚性幹（ES）細胞は、発生初期胚の多能性細胞群より樹立され、体外培養条件下で未分化性を維持したまま無限に自己増殖する。ES 幹細胞の自己増殖はこれまで親細胞が同一な娘細胞に分裂することにより担われていると考えられてきた。しかしながら近年の遺伝子発現の解析から、ES 細胞は単一の細胞集団ではなく不均一な亜集団からなる細胞集団であることが明らかになってきた。

ここで少なくとも挙げられるいくつかの疑問は、

（1） 不均一な細胞集団は何を反映しているのか？

（2） 細胞集団の割合はどのような様式で維持されるのか？

（3） それぞれの細胞集団に機能的な差異はあるのか？

という点である。これらの疑問を解くために我々の研究室では、ES 細胞に不均一に発現する遺伝子 Stellla/Dppa3 に注目した。Stella/Dppa3 の発現は胚発生において未受精卵から胚盤胞の内部細胞塊（ICM）に認められ、着床後 ICM が原始外胚葉（エピブラスト）に発生する段階で消失する。その後 Stella/Dppa3 の発現はエピブラストが原腸陥入した後に現れる始原生殖細胞（PGCs）に再び認められる。Stella/Dppa3 の発現を GFP の発現で可視化できるレポーターマウス ES 細胞を用いた解析の結果、Stella/Dppa3 陽性 ES 細胞には ICM 特異的な遺伝子の発現が認められ、対照的に Stella/Dppa3 陰性 ES 細胞にはエピブラスト特異的な遺伝子の発現が認められた。ES 細胞におけるそれぞれの亜集団の割合は継代数に関わらず一定であった。またそれぞれの亜集団を単離して培養した結果、いずれの亜集団からも他方の亜集団が出現し、最終的には分離前の ES 細胞に占めるそれぞれの割合に戻ることが明らかとなった。またそれぞれの亜集団の分化能は異なり、Stella 陰性 ES 細胞は陽性細胞に比べ、分化を誘導するシグナルに対し高い感受性を示した

これらのことから、

（1） ES 細胞を形成する亜集団は、胚発生の過程にある程度従った遺伝子発現パターンを維持している。

（2） 自己増殖を繰り返す ES 細胞において、それぞれの亜集団がある一定の割合で遷移しながらその平衡状態を保っている。

(3)　それぞれの亜集団は機能的に異なった細胞集団である
ことが示唆された。

これらと類似した自己増殖の様式は他の幹細胞にも認められることから、更なる解析は ES 細胞のみならず、様々な幹細胞の自己増殖様式を知るうえで重要であると考えられる。また亜集団の割合の人為的制御はES細胞に均一な分化を誘導しうるという点で、今後の ES 細胞制御の研究に重要であると考えられる。本講演では以上の結果に加え最近の知見を紹介し、さらに幹細胞からの生殖細胞の分化制御の可能性について討論する。

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会員各位

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テーマ「卵・胚のクオリティ」

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開催日時：2010年3月21日 午後1時開演

開催場所：明治大学 駿河台キャンパス リバティタワー

セッションI

座長　石塚 文平（聖マリアンナ医科大学）

1. 「DHEA内服前後の採卵に対する影響」
   大塩 達弥（東京ベイレディースクリニック）
2. 「ヒトおよびマウスIVM卵のミトコンドリアの動態と卵・胚のクオリティ」
   吉田 仁秋（吉田レディースクリニック）
3. 「哺乳類未受精卵および初期胚の超低温保存に影響を及ぼす諸要因」
   伊藤 潤哉（麻布大）

Coffee break

セッションII

座長　鈴木 秋悦（東京生殖バイオロジー東京シンポジウム）

4. 「マウス体内におけるブタ卵胞の発育と卵の発生能」
   金子 浩之（農業生物資源研）
5. 「胚盤胞の品質評価」
   乾 裕昭（乾マタニティクリニック）
6. 「新しい胚評価法に基づく最適な胚移植時期」
   古井 憲司（クリニックママ）

総合討論 座長　鈴木 秋悦・石塚 文平

懇親会

編 集 後 記

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