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The Production of Egg Yolk Immunoglobulin (IgY) Raised against 3T3L-1 Cell Membrane Protein and the Control of Adipocytes Differentiation

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3T3L-1세포의 막단백질에 대한 난황면역글로불린 (IgY)의 생산과 지방세포의 분화조절작용

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ABSTRACT

The present study was undertaken to establish a model for the control of adipocytes differentiation by using antibody produced from egg yolk. The emulsion of membrane protein of 3T3L-1 cell membrane protein with the complete Freund's adjuvant was firstly immunized in layer. Second and third boosting were undertaken with two weeks intervals by injection of the emulsion of the same antigen with the incomplete Freund's adjuvant. After 4 weeks of the first immunization, eggs were collected and antibody(IgY) was purified from egg yolk. The purity of IgY was 60-98% determined by single radial immunodiffusion (SRID) methods. Titer value of the antibody showed high reactivity for the preadipocytes membrane protein measured by ELISA. When the IgY was added in the test media containing either 2.5% porcine serum or 10% FBS(control), the differentiation of 3T3L-1 cells and Glycerol-3-phosphate dehydrogenase(GPDH) activities was significantly decreased compared to the control cells($p<0.05$). When mice were subcutaneously injected with IgY raised against membrane protein of 3T3L-1 cells for 3 weeks, adipose tissue mass around ovary was tended to be decreased in female mice compared to those of control mice. It is suggested that a potential for manipulating of lipid accumulation through decrease in 3T3L-1 cell differentiation and fat accumulation in female mice by IgY treatment.

(key words : IgY, Antibody, 3T3L-1 cells, Adipocytes Differentiation, fat accumulation, Layer)

INTRODUCTION

Excess fat deposition in livestock is major concern in meat production industry due to high production cost. Extensive investigation was undertaken to reduce the body fat by ways of nutritional (Lhuillery et al., 1988, Becker et al., 1986), hormonal (Warriss et al., 1990), physiological regulations (Dauncey et al., 1983). Sev-

eral success has been achieved through the growth hormone and "β-adrenergic compounds to improve carcass composition (Futter et al., 1990; Warriss et al., 1989). However, periodical implantation is needed in case of growth hormone, and the use of such hormones have undesirable side effects (Warriss et al., 1989, 1990). With these reasons, the use of hormones was already banned in European countries. As an alternative strategies for the control of body fat, last few years, several studies demonstrated that polyclonal antisera raised against adipocytes membrane protein have cytotoxic effect on cell surface of adipocytes (De clercq et al., 1997; Butterwith et al., 1989; Flint et al., 1986). When growing rats and pigs are passively immunized with these antisera, it could induce massive lysis of adipocytes leading to reduction in the proportion of body fat. The antisera production by immunization and bleeding gives animals much stress but only limited amount of antisera is available. It is known that the hen transfers her serum immunoglobulin to the egg yolk and gives immunity to her offspring. It is well known that egg yolk immunoglobulin (IgY) can be quickly and easily isolated from chicken egg yolk (Akita et al., 1992). The practical application of IgY by oral administration for preventing some diseases were recently introduced in the field of passive immunization therapy (Bartz et al., 1980). In this case, a convenient and large-scale supply of IgY may be required.

The present study was undertaken to accomplish the model system for the production of IgY and control of body fat accumulation by using chicken IgY raised against 3T3-L1 cell membrane protein.

MATERIALS AND METHODS

1. Cell Culture

3T3L-1 mouse fibroblast cells were cultured with Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco, Grand Island, NY) in a humidified atmosphere of 5% CO_2. 5×10^6 cells were cultured under test media containing DMEM with 2.5% Porcine serum or 10% FBS(control) and differentiation was induced with insulin (10μg/mL), 3-Isobutyl-1-Methylxanthine (IBMX;0.5mM) and Dexamethasone (0.25μM) with or without IgY (5μg/mL). Testing media was changed every other day for 7-10 days.

2. IgY Production and Purification

For the membrane protein preparation, 6×10^7 3T3L-1 cells were homogenized in 8mL of membrane extraction media(40mM HEPES, 1% TritonX-100, 10% Glycerol, 5μM EDTA, 1mM PMSF, 1mM DTT) by using Dounce homogenizer. The homogenate was centrifuged at 100,000 $\times g$ for 1 hour at 4℃. The pellet was gently suspended in sterilized 0.85% saline solution. After measurement of protein concentration, the membrane protein was stored at −70℃ until they were used.

10 ISA-brown layers, 77 weeks of age, were immunized with 1 ml of emulsion of 250μg of membrane protein and complete Freund's adjuvant. The layers were then boosted twice more at 2 weeks intervals with the emulsion of the same antigens as used in first immunization and incomplete Freund's adjuvant.

The IgY was isolated by the methods of Hatta et al. (1990). Briefly, egg yolk was carefully separated from egg white, washed with distilled water to remove as much albumin as possible and put an egg yolk and roll it on paper towels to remove adhering egg white. Then, the membrane was punctured and the yolk allowed

to flow into a conical tube without any contamination of membrane. Egg yolk was then diluted with same amount of distilled water. The mixture was well mixed with four volumes of 0.15% λ-carrageenan solution per volume of egg yolk and left for more than 15 hours at 4℃ followed by centrifugation at 4,000×g for 15min. The supernatant was filtered through filter paper and ammonium sulfate (saturated) solution was added onto the supernatant until the precipitation was made. After centrifugation at 4,000×g for 15min, the supernatant was decanted and suspend the pellet with 4 times cold PBS of original egg yolk volume. Sodium sulfate anhydrous powder was added into the suspension to be 18% (W/V). The mixture was shaken in shaking water bath until sodium sulfate was completely resolved followed the centrifugation at 4,000×g for 15min. The resulting precipitate was dissolved in twice volume of cold PBS. After filtration with 0.45μm membrane filter, protein concentration was measured for further experiments.

3. Single Radial Immunodiffusion (SRID) Assay

Radial immunodiffusion assay was done by the method of McCannel and Nakai (1990). 1% of Agarose gel containing 2.5mg/mL of Rabbit anti-chicken IgG anti-sera (Sigma Chemical Co., St. Louis, MO) was made and poured into RID plates. 6μl of appropriately diluted samples and standards of 0.1, 0.2, 0.5 and 1.0 mg/mL were added to 3mm diameter wells. IgY concentration of unknown samples was determined by reference of standard curve obtained by plotting of the diameter square of each concentration.

4. Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed as described by Shimizu et al. (1988). Wells were coated with 0.5μg of membrane protein in Carbonate/Bicarbonate buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, 3mM NaN_3, pH 9.8), and incubated overnight at 4℃. Plates were washed three times with PBS-T (0.05% Tween-20), followed by a blocking step using 100μl of blocking buffer (0.17M H_3BO_3, 0.12M NaCl, pH 8.5, 0.05% Tween-20, 0.25% BSA, 0.05% NaN_3) for 30min at 37℃. Plates were incubated with the serial dilutions of purified IgY for 1hr at 37℃. The plates were then washed again three times with PBST and 100μl of rabbit anti-chicken IgG conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, 1:500 in PBST) was added to each well. After 1hr incubation at 37℃, the plates were washed twice with PBST and 50μl of substrate solution (0.1% p-nitrophenyl phosphate disodium in glycine buffer : 0.1M glycine, 1mM $MgCl_2 \cdot 6H_2O$, 1mM $ZnCl_2$, pH 9.8). The reaction was stopped by addition of 50μl of 0.5M NaOH solution. Absorbance was read at 405nm using a ELISA reader (BIO TEC EL311SL).

5. *In vivo* Administration of IgY

Twelve male and six female, weaned at 3 weeks of age, ICR mice (purchased from Korean Food and Drug Administration), were divided into control and IgY group, respectively, and housed in plastic cages containing a saw dust bedding (3 mice per cage) at 21~24℃ and 40~60% relative humidity with 12 hour light/dark cycle. Mice received food (Purina Certified Laboratory Chow) and water *ad libitum* for 3 weeks. Mice were subcutaneously injected 200μl of emulsion of IgY-Freund's incomplete adjuvant (0.05μg/μl) once in a week for 3 consecutive weeks. Body weight gain and the adipose tissues of around ovary and testis were

gathered and weighed after finishing of the experimental feeding.

6. Glycerol-3-phospate Dehydrogenase (GPDH) Activity

Cells were washed twice with cold PBS and harvested by scraping in 0.3ml Tris-EDTA buffer (25mM Tris-HCl, 1mM EDTA, pH 7.5). Cells were homogenized by sonication for 5 seconds and centrifuged at 13,000×g for 5min. at 4℃. The supernatant fluids were used for analysis. Glycerol-3-phosphate dehydrogenase was assayed according to the procedures of Kozac and Jensen (1975).

7. Statistical Analysis

Data were analyzed using the GLM procedures of SAS (1985). Statistical significance of *differences between means were determined* by the least significant difference using SAS or the student's t-test.

RESULTS AND DISCUSSION

1. Yield of IgY and Its Purity

The summary of IgY production and isolation was shown in Table 1. The major components of egg yolk are various lipoprotein, especially low density lipoprotein (LDL) and high density lipoprotein (HDL). In our experiments, 0.15% λ-Carrageenan solution was added into doubly diluted egg yolk for the precipitation of lipoprotein. The final yield of IgY was about 32±2.7mg per egg out of 45±3.7 mg of total IgY of the supernatant fraction with being resulted in the recovery of more than 70%. The purity of IgY was ranging from 60 to 98% detected by the RID methods. Hatta et al. (1990) demonstrated that 70~100mg of IgY was obtained per egg and the purity of IgY was more than 98% after three times salting-out with sodium sulfate. When simple water dilution was used to fractionate water-soluble plasma proteins from egg yolk granules, an optimum recovery of IgY in water soluble fraction was more than 90% (Kwan et al., 1991; Akita et al., 1992). Our data in IgY production showed similar results to these data. When we gathered 5~6 eggs from immunized layer for a week, about 150~180 mg of IgY was available suggesting that a large amount of IgY was able to be easily isolated from egg yolk without giving any stress by bleeding.

2. Activity of IgY

The activity of obtained IgY was measured by ELISA. As shown in Figure 1 and 2, the immunoglobulin isolated from egg yolk raised against the 3T3-L1 preadipocytes membrane protein showed higher titer values even at di-

Table 1. The concentration of egg yolk immunoglobulin (IgY) raised against 3T3L-1 cell

	Total protein	Total IgY	Recovery	Purity of IgY
	(mg/ml)	(mg/ml)	(%)	(%)
Supernatant*	235±10.0	45±3.7		
After desalting	45± 5.0	32±2.7	71.1	60~98

Values are means±SE of 3 replicates. Initial egg yolk was average 13.0 mL.

* Supernatant : The doubly diluted egg yolk was mixed with 0.15 % λ-carrageenan solution(four times of egg yolk) and stood over night in refrigerator. The mixture was then centrifuged at 10,000 × g for 15min. The supernatant was then filtered through a filter paper.

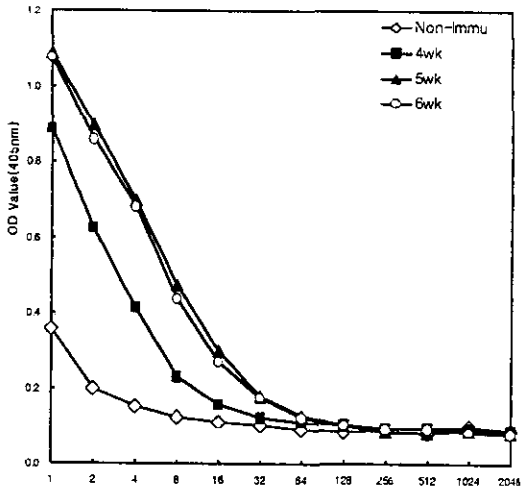

Figure 1. Reactvity of various IgY raised against 3T3L-1 cell membrane proteins toward 3T3L-1 cell membrane proteins as determined by Enzyme-linked Immunosorbent Assay (ELISA).

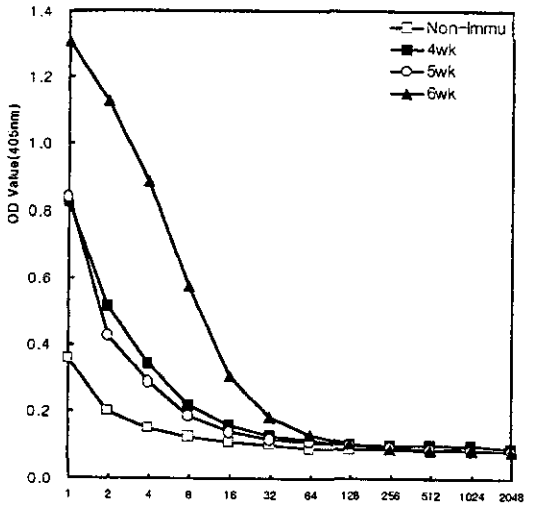

Figure 2. Reactvity of various IgY raised against 3T3L-cell membrane proteins toward 3T3L-1 cell memprane proteins as determined by Enzyme-linked Immunosorbent Assay(ELISA).

lution of 1:2048 (50ng of IgY/well) while non-immunized control IgY showed almost no reactivity toward 3T3-L1 preadipocytes membrane protein. This results suggested that four weeks from first immunization was enough time to produce the active functional IgY.

3. Cell Differentiation and GPDH Activity

Differentiation of 3T3L-1 cells was well induced with insulin (10μg/mL), 3-Isobutyl-1-Methylxanthine (IBMX; 0.5mM) and Dexamethasone (0.25μM) in both test media containing DMEM with 2.5% Porcine serum and 2.5% FBS. When IgY (5μg/mL) was added into the media, cell differentiation was drastically suppressed (photo 1-1, 1-2 and 2-1, 2-2). Under the light microscopic observation, fat droplet was small in both size and number in the cells treated with IgY. Recently, De clercq et al. (1997) have demonstrated that, when well differentiated stromal vascular cells were exposed to anti-adipocyte monoclonal antibody for 24 hours, the antibody was cytotoxic to porcine preadipocytes in the presence of complement in serum containing or in chemically defined serum free media. The cytotoxic effect was not dose dependent at least between 0.1 and 10μg/mL. However, when they treated the cells with complement and antibody before differentiation on day 3, the number of the differentiated cells were fewer than control cells. In our experiment, we treated the cells with antibody (5μg/mL) from the first day of differentiation induction, and at the end of the culture on day 7 the clear suppression of lipid accumulation was found without any cell lysis detected by the light microscope. The effects of IgY raised against the 3T3L-1 cells on GPDH activities in the media containing either FBS or porcine serum are shown in Table 2.

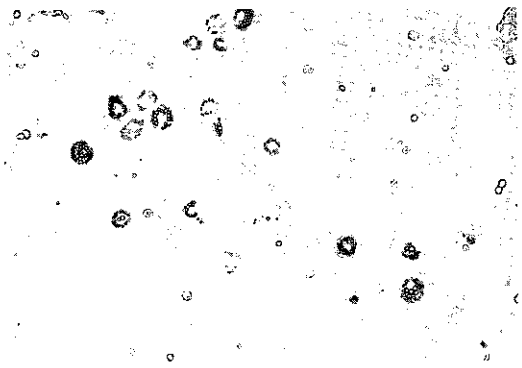

Photo 1-1. The photograph of 3T3L-1 cells differentiated with insulin (10mg/mL), IBMX(0.5mM) and dexamethasone (0.25μM) for 7 days under the medium containing FBS.

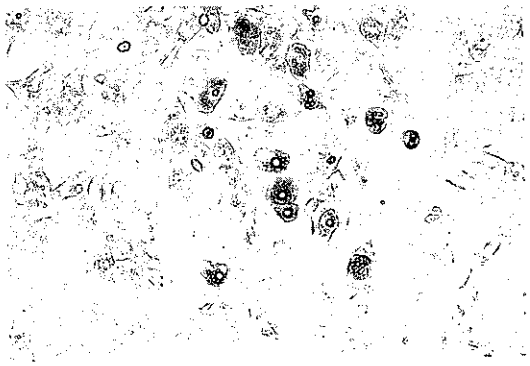

Photo 2-1. The photograph of 3T3L-1 cells differentiated with insulin (10mg/mL), IBMX(0.5mM) and dexamethasone (0.25μM) for 7 days under the medium containing porcine serum.

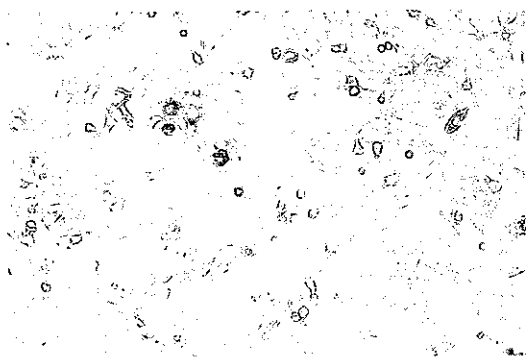

Photo 1-2. The photograph of 3T3L-1 cells differentiated with insulin (10mg/mL), IBMX(0.5mM) and dexamethasone (0.25μM) and IgY(5μg/mL) for 7 days under the medium containing FBS.

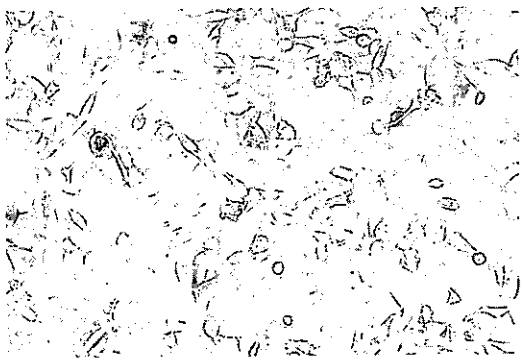

Photo 2-2. The photograph of 3T3L-1 cells differentiated with insulin (10mg/mL), IBMX(0.5mM) and dexamethasone (0.25μM) and IgY(5μg/mL) for 7 days under the medium containing porcine serum.

By the treatment of Dexamethasone(0.25 μM), 3-Isobutyl-1-Methylxanthine (IBMX;0.5 mM) and insulin(10μg/mL), the activities of GPDH were significantly increased under both porcine serum and FBS ($p<0.05$). When the IgY(5μg/mL) raised against 3T3-L1 cells were contained in the differentiation medium, however, GPDH activities were significantly decreased. Similar results were also observed in cells treated with FBS.

In our experiments, when the cells were treated with IgY(5μg/mL), cytotoxicity was not clearly observed but the differentiated cell numbers were decreased and even the different-

Table 2. Effect of IgY raised against the 3T3L-1 cell membrane protein on relative GPDH activities

Treatment	FBS	Porcine serum
	Index to + Ins /FBS	
−Ins	15.000±0.500^{c}	15.000± 0.500^{c}
+Ins	100.000±0.000^{a}	134.900±21.200^{a}
+Ins +IgY	72.300±0.300^{b}	69.800± 1.500^{b}

Values are mean±SE of 3 replicates and determined as mmol /min /μg protein. FBS : Fetal bovine serum
−Ins : without insulin in media
+Ins : with insulin in media
+Ins+IgY : with insulin and IgY(5μg /mL) in media
Differentiation was induced with insulin(10μg /mL), IBMX(0.5mM) and dexamethasone(0.25μM) in the media containing either 10% FBS or 2.5% porcine serum for 7 days with or without antibody.
a~c : Means with a column with no common superscript differ significantly at $p<0.05$.

iated adipocytes was not fully filled with fat drops. It is still not known if adipose conversion can be down-regulated through non-cytotoxic mechanism, such as cell surface protein signaling transduction. Further studies are needed to clarify this point.

4. Adipose Tissue Pad Weights

For *in vivo* administration, IgY was newly produced against the membrane protein of 3T3L-1 cells.

When mice were passively immunized with IgY by subcutaneous injection once in a week for 3 consecutive weeks, there was no difference in epididymal adipose tissue pad weights per body weight gain between control and IgY-injected group mice (Table 3). In female mice, however, the ovary adipose tissue pad weights per weight gain tended to decrease in mice passively immunized with IgY. It has been reported that a reduction of body fat accumulation was found with *in vivo* administration of anti-sera (anti-adipocyte antibodies) in rats (Parton et al., 1990; Hu et al., 1992), pigs (Kestin et al., 1993) bovines (Cryer et al., 1984), chicken (Dong et al., 1991; Butterwith et al., 1989, 1992a,) and sheep (Nassar and Hu, 1991). Oral administration of immunoglobulins

Table 3. Effect of injection of antibody emulsion on the body weight gain and adipose tissue pad accumulation in mice

	Female		Male		SEM	LSD
	Control	+Ab	Control	+Ab		
Body weight						
Final g /head	27.92^{b}	27.12^{b}	34.58^{a}	35.12^{a}	0.94	2.14
Gain g /period /head	4.30^{b}	5.10^{b}	8.20^{a}	7.90^{a}	0.41	2.50
Adipose pad						
g /BW	0.453^{b}	0.307^{b}	0.640^{a}	0.683^{a}	0.046	0.199
g /100g BW	1.631^{ab}	1.095^{b}	1.795^{ab}	1.940^{a}	0.124	0.578
g /Gain	0.105^{a}	0.060^{b}	0.078^{ab}	0.087^{ab}	0.008	0.034

Values are means of 3 replicates(head).
SEM : standard error of mean
LSD : Least significant difference
+Ab : Injected the emulsion of IgY against 3T3L-1 cell membrane proteins with incomplete freund's adjuvant
a~b : Mean with a row with no common superscript differ significantly at $p<0.05$.

from chicken egg had been used successfully for the prevention of murine rotavirus infection in mice (Bartz et al., 1980). However, no results of administration of IgY to *in vivo* administration for the manipulating carcass fat content were found. In order to establish a model system for control of fat accumulation, we produced IgY raised against the membrane protein of 3T3L-1 cell line originated from mouse fibroblast and injected subcutaneously to mice. There were no impairment in body weight gain by injection of IgY showing decreasing tendency in lipid accumulation. This finding may be very encouraging to introduce this system for the manipulation of adipose tissue growth in pig industry as well as humans.

적 요

본 연구는 산란계를 이용하여 지방세포 분화조절을 위한 다량의 난황항체를 생산하는 모델을 세우는데 그 목적이 있다. 항원인 250μg의 지방전구세포(3T3L-1) 막단백질과 complete Freund's adjuvant와 잘 혼합한 유화액 1mL를 가슴부위의 근육내 250μl씩 네 곳에 나누어 주입하였다. 2주 간격으로 2차 및 3차에는 동일항원과 incomplete Freud's adjuvant로 만든 에멀젼을 주사하였다. 항원 주사후 4주부터 6주에 걸쳐 수집한 계란에서 항체를 분리하였다. 단일원면역확산법(Single Radial Immunodiffusion; SRID)에 의해 측정된 난황항체의 순도는 60~98%가 되었다. 생산된 난황항체의 역가는 Enzyme Linked Immuno-Sorbent Assay (ELISA)법에 의해 측정하였다. 작제된 항체를 지방전구세포(3T3L-1 cell)와 반응시켰을 때 면역원을 주입하지 않은 계란으로부터 분리한 항체는 거의 반응을 나타내지 않았다. 이에 비해 3T3L-1 막단백질을 주입하여 얻은 항체는 높은 역가를 나타내었으며, 최초 100μg의 항체를 사용하였고 2,000배 이상의 희석배수에서도 특이적 반응이 관찰되었다. 이렇게 특이성을 갖는 항체의 세포내에서의 작용을 조사하기 위하여 직접 인슐린에 의해 분화중인 3T3L-1 세포주를 2.5%의 돼지혈청(porcine serum) 혹은 10%의 FBS(대조)를 함유하는 DMEM 배지에 항체를 처리한 후 Glycerol-3-phosphate dehydrogenase(GPDH) 활성에 미치는 영향을 조사하였다. 항체를 처리한 분화중인 3T3L-1세포에서는 대조세포에서의 GPDH활성보다 유의하게 낮았다 ($p<0.05$). 본 실험결과는, 높은 역가를 나타내는 난황항체처리로 지방전구세포의 분화가 억제되며 암마우스에서는 지방조직의 축적을 감소시키므로, 가금항체에 의한 포유류의 지방축적조절 가능성을 나타내는 것이다.

(주제어 : 난황면역글로뷸린, 항체, 3T3L-1세포, 지방세포분화, 지방축적, 산란계)

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