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Protein Profile of Embryonic Stem Cells Following Differentiation

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ABSTRACT

To investigate the expression patterns of proteins and growth factor signals in differentiated rabbit embryonic stem (ES) cells, ES cells with confluent stage grown of feeder layer and differentiated cells into embryoid bodies (EB) without feeder cell were applied to protein gel and Western blotting analysis. There were 66kDa and 28kDa specifically expressed in differentiated ES cell but not in undifferentiated ES cell while 25kDa protein band showed up in only undifferentiated ES cells. Also there were some difference of protein bands in several area of gel between differentiated and undifferentiated ES cells such as about 100 kDa, 50kDa and 27kDa areas, but there was no difference in band pattern of one-dimensional gel analysis between mouse ES cells and rabbit ES cells. IGF-I receptor and EGF receptor were expressed in differentiated cells and undifferentiated cells. And IGF-I and EGF were not expressed in both differentiated and undifferentiated cells. These results indicated that ES cells express their own proteins to inhibit differentiation while EB cells synthesize different proteins to differentiate, and IGF-I receptor and EGF receptor were expressed in both ES and EB cells probably for the different functions.

(Key words : Embryonic stem(ES) cell, Differentiation, Embryoid body (EB))

Ⅰ. INTRODUCTION

The establishment in culture of pluripotent embryonic stem (ES) cells derived from embryos has ushered in an exciting new era in the study of developmental molecular genetics (Cappecchi 1989). These cells, originally derived from the proliferating inner cell mass of preimplantation embryos (Evans and Kaufman, 1981; Martin, 1981), can be proliferated indefinitely in an undifferentiated state. Much of the recent interest in ES cells has derived from

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their use as a vehicle for the introduction of genetic alterations into the animal germline.

The formation of embryoid bodies (EB) by aggregation of ES cells in suspension leads to differentiation of the pluripotent cells in an ordered and predictable manner that shares many features with mouse early embryogenesis (Doetschman et al., 1985). Outer cells of the body acquire the fate of primitive endoderm and, over a period of some days, its differentiated derivatives visceral and parietral endoderm. This is the normal fate of externally positional cells of the pluripotent ICM in the embryo (Gardner, 1983). Gene expression within the central pluripotent cells could be shifted from an ES/ICM state to primitive ectoderm. This is accompanied by formation of an internal cavity in a series of events analogous to formation of the proammniotic cavity and primitive ectoderm *in vivo* (Shen and Leder, 1992). Differentiation of primitive ectoderm within the body results in formation of partially and terminally differentiated cell types in which derivatives of all three germ layers can be detected (Doetschman et al., 1985).

There have been reports of ES or ES-like cell lines from a variety of mammals, but few of these have been validated extensively. Some of these lines are capable of differentiation into derivatives of the three primary germ layers *in vitro*, and/or retain some pluripotent cell-specific gene expression (Pederson, 1994). It is particularly exciting that pluripotent cells capable of contribution to chimeras have now been reported from chicken (Pain et al., 1996), rat (Iannaccone et al., 1994) and pig (Shim et al., 1997). However, the ability of these lines to retain pluripotence following extended periods of culture *in vitro*, or genetic manipulation and expression of clonal cell lines, has not been demonstrated clearly.

Different cell markers have been used to characterize undifferentiated versus differentiated ES cells. These markers generally correspond to (glyco) proteins of the cytoskeleton, cell membrane or extracellular matrix demonstrated by immunocytochemical techniques. Markers may also be revealed by their eventual enzymatic activity. They are several classical positive markers of undifferentiated ES cells. Among them alkaline phosphatase (Wobus et al., 1984) which is equivalent to the cell surface non-specific alkaline phosphatase of the inner cell mass of the mouse blastocyst (Mulnard and Huygens, 1978) is commonly used as a stem cell marker.

The rabbit satisfies factors considering when selecting an animal species for these studies, such as ease in housing, manipulation of embryos, litter size, cost to maintain; its size and physiology have made it a favorite for numerous types of investigations for decades. It has a relatively short gestation period of 31 days (compared to 19 days for the mouse) and generally reaches sexual maturity by 5-7 months of age. In addition, the females are induced ovulators (the act of coitus induces ovulation) and conditions for superovulation have been well defined (Kennelly and Foote, 1965).

The purpose of this study is to investigate protein profile of isolated rabbit ES cells compared to differenciated cells. For this goal, these stem cells were cultured without feeder layer and identified to be formed differentiated embryoid body. Differentiated and undifferentiated ES cells were compared in protein expression pattern by SDS -PAGE and examined to express IGF-1 and EGF and their receptors binding specific antibodies by Western blotting.

Ⅱ. MATERIALS AND METHODS

1. Isolation of ES Cells

When completely hatched embryos were attached on feeder cells trophoblast cells with differentiated

appearance were removed and immediately cells of inner cell mass were placed individually onto mitomycin C treated mouse embryonic fibroblasts (MEFs) feeders in 96-well culture dishes. Hatched embryos generally attached within 48h and were then fed every other day with fresh ES media (consisting of high-glucose DMEM supplemented with 15% FBS, 1% penicillin and streptomycin, 1% glutamine). On day 7 following placement in culture, cells of inner cell mass line were trypsinized and then stem cell colony was isolated by passaging culture. By expanding culture from 96-well plate and to 6-well plate, colonies were harvested and preserved the cell line by freezing.

2. Formation of Embryoid Body

Stem cells grown in confluent state were slightly trypsinized and colonies were transferred to ungelatinized dish (bacteria culture dish) on undestroyed codition. Colonies were cultured for 5~7 days in DMEM medium with 15% FBS at 37℃, 5% CO_2.

3. Protein Pattern Analysis of Differentiated and Undifferentiated ES Cells

1) Protein Extraction

ES cells in confluent state grown on feeder layer and differentiated cells into embryoid body without feeder layer were treated with lysis buffer (0.3% SDS, 1%(v/v) β-mercaptoethanol, 50mM Tris-HCl (pH 8.0)) and cell lysates were centrifuged for 10min at 4℃, 15,000g. Supernatant was transferred into new tube and preserved in −70℃ deep freezer until use.

2) SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Electrophoresis were carried out using Mini-Protean II apparatus (Bio-Rad). After protein extract and sample buffer was mixed, sample was boiled for 5min. The gel was used 12% resolving gel (52% acrylamide, 1,4 bis-acrylamide ; 1.5M Tris-HCl (pH 8.0), 0.4% SDS; 1.4% ammonium persulfate; 0.1% TEMED) and 10% stacking gel (52% acrylamide, 1.4% bis-acrylamide; 0.3M Tris-HCl (pH 8.0), 0.4% SDS; 1.4% ammonium persulfate; 0.1% TEMED). Running buffer (0.2M Glycine; 25mM Tris-HCl (pH 8.0); 4.3mM SDS) of pH 8.3 was used. Electrophoresis was running one gel per 60V for 70min. Molecular weight standards were used Low Range Standards of Bio-Rad and their molecular weight was Phosphorylase b (97.4kDa), Bovine serum albumin (66.2kDa), Ovalbumin (45.0kDa), Carbonic anhydrase (31.0kDa), Soybean trypsin inhibitor (21.5 kDa), Lysozyme (14.4kDa).

The protein pattern was analyzed by silver staining. For silver staining, gel was shaken at least 1h in fixing buffer (50% methanol; 12% acetic acid; 0.5ml/L 37% formaldehyde) and then washed for 20min in 50% ethanol. The gel was incubated in 0.2g/L sodium thiosulfate for 1min, followed by washing three times for 20sec with distilled water and stained with staining buffer (2g/L $AgNO_3$; 0.75 ml/L 37% formaldehyde) for 20min. After staining, the gel was washed three times with distilled water for 20min and then in developing buffer (60g/L Na_2CO_3; 0.5ml/L 37% formaldehyde; 4ml/L $Na_2S_2O_3$) with shaking. During shaking, when the bands were appeared, the gel was fixed with 1% acetic acid. Staining buffer and developing buffer was used with distilled water, and buffer was made just before using. Particularly, after making developing buffer, it was used at 4℃.

4. Western Blotting

Blotting was carried out using Mini Trans-Blot Electrophoretic Transfer Cell of Bio-Rad.

Following electrophoresis, polyacrylamide gel and PROTRAN nitrocellulose transfer membrane (Schleicher & Schuell) was sandwiched for electro-

blotting. Fiber pad was placed on transfer cassette and filter paper (Whatman 3MM paper), gel, membrane, filter paper, and filter pad were put. With care not to enter air bubble in the membrane. After putting transfer buffer, the power supply was set to 150V and transferred for 1h. Molecular weight standards used in this experiment were Broad Range Prestained Standards of Bio-Rad and their molecular weights were Myosin (205.0kDa), (-galactosidase (120.0kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (52.2kDa), Carbonic anhydrase (36.3kDa), Soybean trypsin inhibitor (30.2kDa), Lysozyme (21.9 kDa) and Aprotinin (7.4kDa).

After transferring, the membrane was incubated in blocking buffer (5% non-fat dry milk) for 1h. The membrane was washed six times for 10min with wash buffer (0.1% Tween 20; 50mM Tris-HCl (pH 7.6); 200mM NaCl; 1mg/ml PEG 20,000; 1.25ml Fetal bovine serum) and was rocked gently with 1:1000 diluted primary antibody (IGF-I, IGF-I receptor, EGF, EGF receptor) for 1h. Wash buffer was washed six times for 10min and then with 1:1500 diluted secondary antibody (Horseradish peroxidase anti-mouse antibody) for 1h. It was washed six times for 10min with washing buffer. The next detection procedure was carried out in a dark room. An equal volume of detection solution 1 and 2 in ECL kit (amersham pharmacia biotech) were mixed (detection reagent). The detection reagent was poured to the membrane on wrap and incubated for 1min without agitation. Detection reagent was drained off and the membrane was wrapped. The membrane was picked in the film cassette with a sheet of Diagnostic film (Kodak) on top and exposed.

Ⅲ. RESULTS AND DISCUSSION

1. Protein Profile of Differentiated Rabbit ES Cell

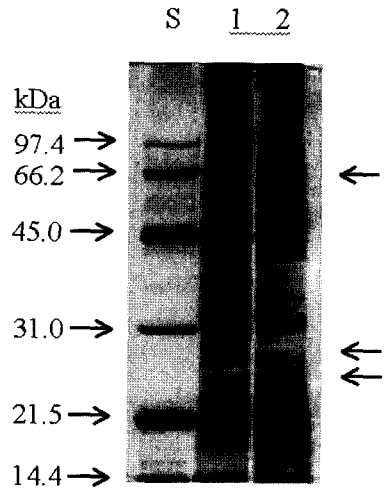

Fig. 1. Analysis of protein band patterns in rabbit ES cells by SDS-PAGE. Lane S, low molecular weight standard; Lane 1, differentiated ES cells (EB); Lane 2, undifferentiated ES cell; Arrows, specific bands in ES or EB.

When rabbit ES cells were cultured without feeder cell, ES cells were differentiated and formed embryoid bodies (EB). To investigate the differential expression pattern of proteins between the differentiated and undifferentiated ES cells, one-dimensional protein gel electophoresis was applied and compared the difference of protein band pattern. The cell proteins were extracted by using lysis buffer, and total protein was separated by SDS-PAGE electrophoresis. The gel following electrophoresis was stained with coomassie blue staining and silver staining methods. There were 66kDa and 28kDa specifically expressed in differentiated ES cell but not in undifferentiated ES cell while 25kDa protein band showed up in only undifferentiated ES cells (Fig. 1). Also there were some difference of protein bands in several area of gel between differentiated and undifferentiated ES cells such as about 100kDa, 50kDa and 27kDa areas, Fig. 2 represents protein band patterns of mouse ES and rabbit ES cells, but

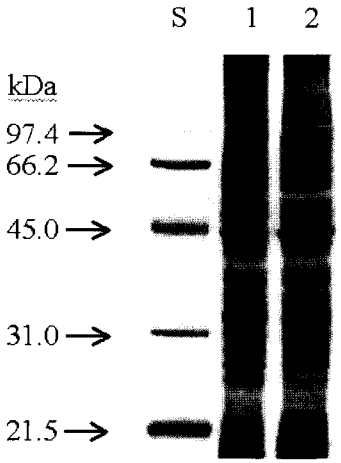

Fig. 2. Analysis of protein band patterns in mouse ES and rabbit ES cells by SDS -PAGE. Lane S, low molecular weight standard; Lane 1, undifferentiated mouse ES cell; Lane 2, undifferentiated rabbit ES cell.

there was no difference in band pattern of one-dimensional gel analysis between them.

All house keeping genes and genes keeping in undifferentiated states probably are expressed in ES cells. There were several transcription factors identified specifically in ES cells such as Oct-3/4, Oct-5 and Oct-6 which are known to activate or repress specific genes involved in proliferation and differentiation of ES cells (Suzuki et al., 1990). Following differentiation, genes involved in developmental regulation could be expressed. Therefore, gene expression pattern between ES and EB cells could be much more complex in activation and repression of genes as well as level of gene expression than in this experiment studied by one dimensional gel analysis. However, this study will contribute to sort out expression profiles of main proteins during differentiation in ES cells that can be used as markers to identify of undifferentiated ES cells for isolating ES cells of animals.

2. Western Blot Analysis

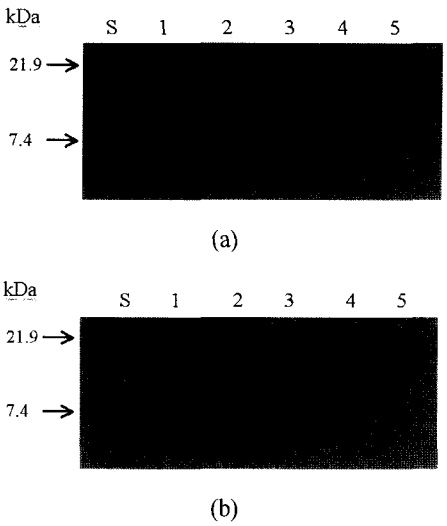

Fig. 3. Western blot analysis using IGF-I(a) and EGF(b) antibody in rabbit ES cells. Lane S, broad range prestained standard; Lane 1, liver; Lane 2, differentiated ES cell (5days); Lane 3, differentiated ES cell (11 days); Lane 4, undifferentiated ES cell (4 days); Lane5, undifferentiated ES cell (10 days).

To examine expression of IGF-I (insulin-like growth factor-I), EGF (epidermal growth factor), IGF-I receptor, and EGF receptor in undifferentiated and differentiated ES cells, Western blotting was carried out with their specific antibodies. ES and EB cells were cultured at intervals of about five days and their protein extracts were applied to western blotting. The proteins of ES and EB cells were separated in size by SDS-PAGE followed by transferring to nitrocellulose membranes and then primary and secondary antibodies were applied on them. Primary antibodies used in this experiment were IGF-I, IGF-I receptor, EGF, and EGF receptor monoclonal antibodies.

The levels of IGF-I and EGF proteins could not be detected in ES or EB cells (Fig. 3). However, IGF-I receptor and EGF receptor were expressed in ES and EB cells (Fig. 4 and Fig. 5). The pattern of

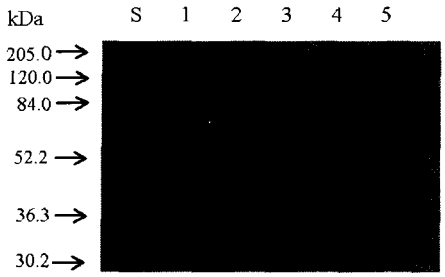

Fig. 4. Western blot analysis using IGF-I receptor antibody in rabbit ES cells. Lane S, broad range prestained standard; Lane 1, liver; Lane 2, differentiated ES cell (5 days); Lane 3, differentiated ES cell (11 days); Lane 4, undifferentiated ES cell (4 days); Lane 5, undifferentiated ES cell (10 days).

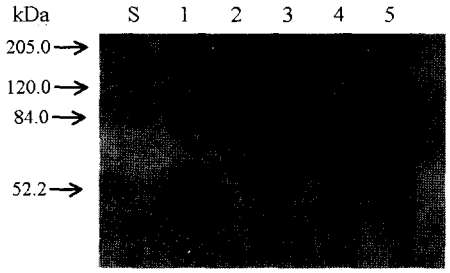

Fig. 5. Western blot analysis using EGF receptor antibody in rabbit ES cells. Lane S, broad range prestained standard; Lane 1, liver; Lane 2, differentiated ES cell (5 days); Lane 3, differentiated ES cell (11 days); Lane 4, undifferentiated ES cell (4 days); Lane 5, undifferentiated ES cell (10 days).

IGF-I receptor was about 79kDa main band with 60kDa and 50 kDa subunits, while EGF receptor was detected with 100kDa, 80kDa, 70kDa, 60kDa (main band) and 50kDa bands. And IGF-I receptor in mouse and rabbit ES cells were expressed with same patterns even though level of expression in IGF-1 receptor subunits was different between mouse and rabbit ES cells (Fig. 6).

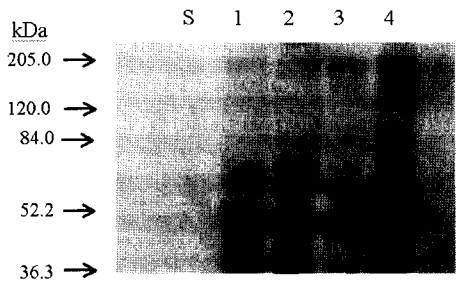

Fig. 6. Western blot analysis of mouse ES and rabbit ES cells using IGF-I receptor antibody. Lane S, broad range prestained standard; Lane 1, differentiated ES cell in mouse; Lane 2, undifferentiated ES cell in mouse; Lane 3, differentiated ES cell in rabbit; Lane 4, undifferentiated ES cell in rabbit.

ES cells synthesize several growth factors of FGF family and TGF ((Health et al., 1989; Slager et al., 1993). Also, several receptors are expressed in ES cells such as LIF receptors, retinoid X receptors and growth hormone receptors (Ohlsson et al., 1993; Bain and Gottlieb, 1994; Ware et al., 1995). Those growth factors and receptors could allow ES cells to maintain undifferenciation and proliferation. IGF-1 and EGF-1 are not secreted in ES cells or EB cells while their receptors are expressed in both cells. These growth factor signals may be utilized for inhibition of differentiation in ES cells, but in EB cells on the other hand they may be activated for differentiation of ES cells to specific tissues. Serum or feeder cells are supply of those growth factors during *in vitro* culture.

In conclusion, these results indicated that ES cells express their own proteins to escape differentiation compared with EB cells and IGF-I receptor and EGF receptor were expressed in both ES and EB cells probably for the different functions.

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