Korean J. Zool. 35:29-36, 1992

Identification of a Fusion-associated Protein in the Skeletal Myoblast Using Monoclonal Antibody

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The present study describes the production of monoclonal antibodies against cultured chick myoblast to pursue critical proteins in muscle cell fusion. Among a panel of monoclonal antibodies, three, MII-3H13, MII-3H18 and MII-3H35 were inhibited moyblast fusion. A single 101-kDa antigen reactive with monoclonal antibody MII-3H35 was detected by radioimmunoprecipitation or by immunoblotting. During the course of myogenesis, the level of the protein remarkably decreased as the cells were differentiated. These results suggest that the protein plays a direct role in the process of myoblast fusion mechanism.

KEY WORDS: Myoblast, Cell fusion, Differentiation, Monoclonal antibody.

A crucial goal of studies in cell differentiation is to discover a single central principle applicable to most or all cellular systems. The appeal of myogenic differentiation as a model system for development from an experimental viewpoint is the ability to induce the conversion of myoblastic cells into myotubes by a relatively short exposure to serum-deficient medium which, in skeletal muscle cultures, results in the fusion of monocytes into multinucleate syncytia (Konigsberg, 1963; Ha *et al.*, 1979; Rosenthal, 1989). Accompanying these morphological changes, various muscle specific proteins such as myosin and actin are synthesized and accumulated with a distinctive pattern which is characteristic of each protein (Buckingham, 1977; Ha *et al.*, 1983).

Many investigations have been focused on the changes of surface proteins and their functional roles during muscle cell differentiation (Hynes *et al.*, 1976; Moss *et al.*, 1978; Walsh and Phillips, 1981). A number of studies have demonstrated that changes in cell surface proteins can be correlated with the modulation of cell to cell interaction (Rosen, 1973; Geltosky *et al.*, 1976). Therefore, phenomena which take place at external cell surface proteins may play a crucial role in the muscle cell membrane fusion process.

The development of immunological techniques has been stimulated by the ability to obtain specific antibodies to membrane antigens even when the latter have not been fully purified or characterized. The problem of obtaining specific antibody without pure antigen (Ag) was solved by the monoclonal antibody (MAb) technique which allowed the isolation of antibodies against individual components of complex epitopes (Köhler and Milstein, 1975). Monoclonal antibodies have been applied in the purification of cell surface antigens (Parham, 1979; Sunderland *et al.*, 1979; Haskins *et al.*, 1983; Kaye *et al.*, 1983; Meuer *et al.*, 1983).

In the present paper, we describe production of a new monoclonal antibody against myoblast

The present study was supported by the Basic Science Research Institute program, Ministry of Education.

which inhibit the fusion of myoblast and identification of its antigen.

Materials and Methods

Primary Cell Culture

Myoblast from breast muscle of 12-day-old chick embryos were prepared according to the method of Ha *et al.* (1979). The cells were plated on collagen-coated culture dishes at a concentration of 5×10^5 cells/ml in Eagle's essential medium (MEM) supplemented with 10% horse serum, 10% chick embryo extract and 1% antibiotic/atimycotic solution. One day after the cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract.

Monoclonal Antibody Production

BALB/c mice were immunized by five intraperitoneal injections of 48 hr-cultured cells of breast muscle (30-50 μg) at intervals of 7 days. A sixth injection with the same immunogens was carried out 21 days later. Three days after this boosting, the splenocytes of the immunized mouse were fused with P3U1 mouse myeloma cells (ratio 10:1) according to a slight modification of technique of Köhler and Milstein (1975). The splenocytes and the myeloma cells were fused in 0.5 ml 40% polyethylene glycol in Dulbecco's modified Eagle medium (DME). For selection of hybridomas, the cells in suspension were distributed in 96-well tissue culture plates containing feeder cells (murine macrophages) and grown in hypoxanthine-aminopterine-thymidine medium. The selective medium was renewed every 2 or 3 days. For screening, undiluted supernatants of growing hybridomas were tested for their ability to bind to 48-hr cultures of muscle cells by the enzyme-linked immunoadsorbent assay (ELISA) and added to myoblast culture medium one day after myoblast seeding for their effects on myoblast differentiation. After screening, positive hybridomas were cloned three times by the limiting dilution technique. Ascitic fluids were obtained by intraperitoneal injection of a chosen clone to pristane-primed BALB/c mice.

Purification of Monoclonal Antibody

Anti-mouse IgG-agarose column (Sigma, A-6531) was equilibrated with 10 mM Tris-HCl/0.5 M NaCl, pH 7.5 (binding buffer). Hybridoma supernatant were adjusted the NaCl concentration to 0.5 M and were clarified by centrifuging at 20,000 $\times$ g and filtering through a 0.45 μm filter. The column was loaded with the supernatant solution and washed with 10 bed volumes of binding buffer. The antibody was eluted with 10 bed volumes of 0.1 M glycine/0.15 M NaCl, pH 2.5, and 0.5 ml fractions of eluant were collected into tubes containing 50 μl 1 M Tris-HCl, pH 8.0. Eluate absorption was monitored at 280 nm with a REC-481 (Pharmacia) fitted with an event marker.

Creatine Kinase and Acetylcholine Receptor Assay

Creatine kinase (CK) assay was perfomed by spectrophotometric method using the hexokinase and glucose-6-phosphate dehydrogenase, as a coupling enzymes (Szasz *et al.*, 1976).

Acetylcholine receptor assay by [^{125}I]-labeled bungarotoxin binding was performed by a modified procedure of Merlie *et al.* (1977). At indicated time, media is removed, cells were washed with MEM for three times. Cells were then preincubated with 1% bovine serum albumin (BSA) in MEM for 30 min in order to reduce the nonspecific binding. Cells were then incubated with ^{125}I-bungarotoxin for 1 hr at 37°C After incubation, cells were washed with PBS three times and dissolved in 1% SDS solution. Finally, toxin binding was measured in a liquid scintillation counter (Packard, TRI-CARB 2000 CA). All measurement were made in triplicate.

Metabolic Labeling and Immunoprecipitation

Thirty six hr later the myoblast cultures were radiolabeled for 12 hr with [^{35}S]methionine (100 μCi/ml) in 2 ml of methionine-free Eagle's medium per 100 mm tissue culture dish. Cells were harvested at 48 hr after plating and lysed by the addition of lysis buffer (RIPA, 50 mM Tris-HCl /1% NP-40/0.5% deoxycholate/1 mM phenylmethylsulfonyl fluoride/10 μg ml^{-1} pepstatin A/

0.1% SDS, pH 7.4). Cell extracts were clarified at 10,000 × g for 10 min and preabsorbed with 160 μl of protein G-bearing *streptococcus* cell suspension (10%, wt/vol) for 30 min at 4°C. For immunoprecipitation, preabsorbed cell extracts were precipitated with 8 μg of monoclonal antibody followed by 160 μl of *streptococcus*. The immunoprecipitated proteins were analyzed by SDS/polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels (Laemmli, 1970). Gels were fluorographed (Bonner and Laskey, 1974) and autoradiographed. Quantitation was achieved by excising the labeled antigen band: gel slices were extracted with 2 ml of 30% H_2O_2/1% HN_4OH for 24 hr at 37°C and radioactivity was counted in 20 ml of scintillation cocktail. [^{35}S] Methionine incorporation into total cell protein was determined by precipitating duplicate aliquots of each cell extract with 20% trichloroacetic acid using 50-100 μg of bovine serum albumin as a carrier.

Immunoblot Analysis

Immunoblot analysis was performed by the semi-dry method (Kyhse-Anderson, 1984). The proteins separated by SDS-PAGE were transferred to nitrocellulose membrane (0.45 μm) with a current of 0.8 mA/cm^2 of gel for 90 min using a transfer apparatus (Hoefer, TE-70). Transferred proteins were stained with india ink (Hancock and Tsang, 1983) and antigen was detected with the anti-myoblast MAb and anti-mouse-IgG conjugated with peroxidase. Color reaction was carried out using bromochloroindolyl phosphate and nitro blue tetrazolium as the substrate.

Results

Production of MAb MII-3H35

The MAbs employed in these studies were generated by immunizing mice with cells from cultures of chick embryo breast muscle. We identified 735 growing hybridomas from original culture wells. Preliminary screening with the ELISA revealed that 42 of these were producing antibodies reactive with the 48-hr cultured myoblasts used for immunization. The supernatants of these positive hybridomas were add to myoblast culture medium for their effects on myoblast differentiation. Three of these hybridomas, designated MII-3H13, MII-3H18 and MII-3H35, was cloned three times by limiting dilution, and once in soft agar. MAb MII-3H35 did not bind to potein A but bind to protein G.

Effect of MAbs on Myogenic Differentiation

Myoblasts in culture seeded at 0.4 × 10^5 cells/ml under standard culture conditions (MEM/10% horse serum/2% embryo extract) undergo rapid fusion during the second day post-plating (Fig. 2). Alignment in these cultures was extensive by 48 hr and fusion was almost complete at 72 hr. The effect of differential concentrations of MAb MII-3H35 on myoblast fusion was determined (Fig. 1). Addition of 1 μg/ml inhibited the fusion slightly, 2 μg/ml inhibited markedly and 3 μg/ml inhibited completely.

Addition of 3 μg/ml MAb, MII-3 and MII-3H35, to the myoblast cultures 24 hr after initial plating, inhibited the alignment and fusion of myoblast (Fig. 2). Action of the MAbs was sustained for an extended period. Growth media of hybridoma exhibited no such activity.

All tested aspects of myoblast differentiation were inhibited by MAb of selected clones, MII-3H13, MII-3H18 and MII-3H35. CK activity and bungarotoxin binding were decreased by the MAbs (Fig. 3).

Detection of Ag MII-3H35

Ag MII-3H35 was isolated from [^{35}S] methionine-labeled cells by specific immunoprecipitation and was analyzed by electrophoretic methods. As shown in Fig. 4, a single band was obtained upon SDS-PAGE. The apparent molecular weight of the material, approximately 101-kDa under reducing conditions.

During the course of myogenesis, the level of the antigen and the synthesis rate of antigen remarkably decreased as the cells were differentiated (Fig. 5). The level of antigen was analyzed by immunoblot of myoblast proteins of 24 hr, 48 hr, 72 hr and 96 hr after seeding. The synthesis rate of antigen was estimated by metabolic labeling of newly synthesized proteins with [^{35}S] and immunoprecipitation of antigen. Particularly antigen fraction of newly synthesized proteins sharply de-

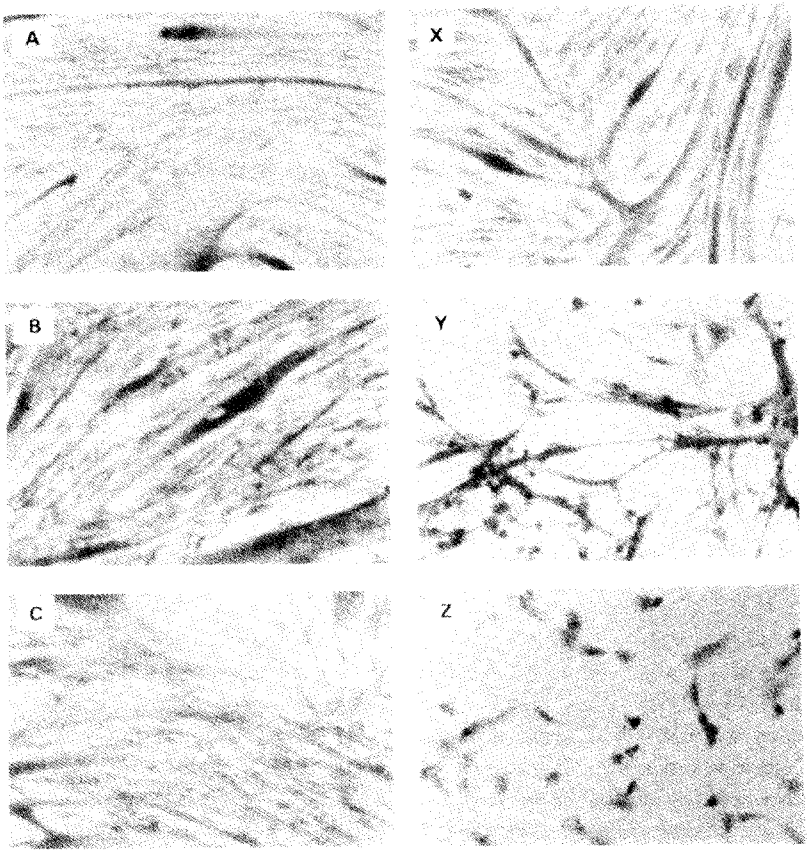

Fig. 1. Effects of differential concentrations of MII-3H35 culture supernatant on myoblast fusion in culture. Cells in normal medium (A), cells in medium with none-coditioned hybridoma growth medium (B, 17%; C, 25%) as blank, cells in medium with MII-3H35 culture supernatant (X, 1 μg/ml; Y, 2 μg/ml; Z, 3 μg/ml) for 72 hr after plating. $\times$ 200.

creased at 48 hr after plating.

Discussion

This report describes the production of monoclonal antibodies directed against cultured chick myoblast to trace some external surface proteins playing a critical role in the muscle cell fusion. In the bioassay after the cell hybridization and single cell cloning, hybridoma culture supernatants were tested against myoblast in culture. This allowed the identification of three clones of hybridomas, i.e., MII-3H13, MII-3H18 and MII-3H35, those secreted antibodies with apparent specificity for the myoblast. And their antibodies inhibited fusion of myoblast completely.

Our monoclonal antibody developed and used in these studies did not bind to protein A from *Staphylococcus aureus* (BRL, 9321SB) with/without anti-mouse IgG developed in rabbit (Sigma, M-6024) as a linker (data not shown), and similar phenomena from certain mouse monoclonal antibodies have been reported (Allison *et al.*, 1982; Schmidt *et al.*, 1985). The affinity of protein A for mouse IgG_1 is very weak (Ey *et al.*, 1978).

In this investigation, we have identified a 101-kDa myoblast protein as antigen of MII-3H35. The function of the protein is currently unknown. In view of the fact that a striking de-

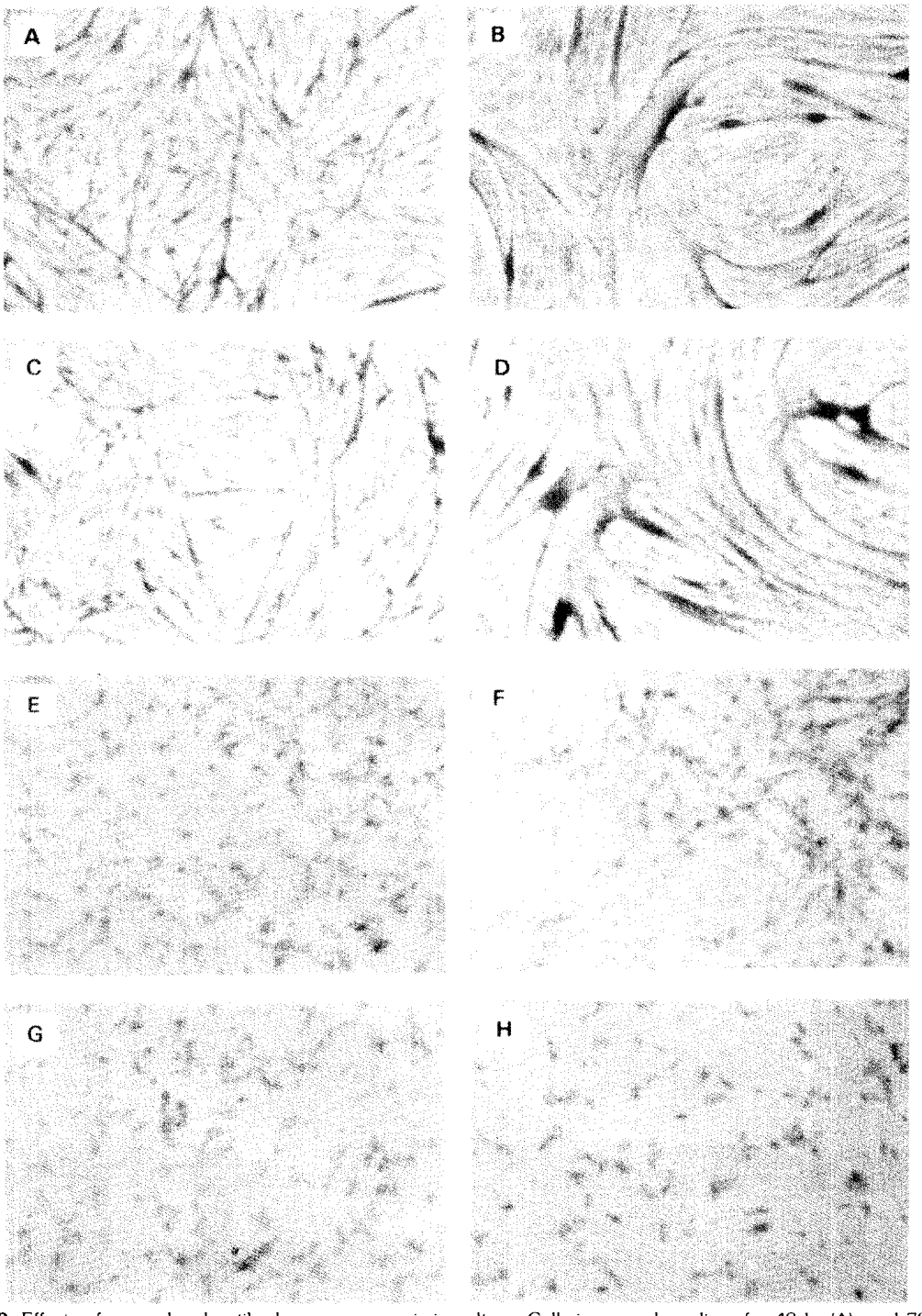

Fig. 2. Effects of monoclonal antibody on myogenesis in culture. Cells in normal medium for 48 hr (A), and 72 hr (B), cell in medium with none-conditioned hybridoma growth medium as blank for 48 hr (C), and 72 hr (D), cells in medium with MII-3 culture supernatant (3 μg/ml) for 48 hr (E), and 72 hr (F), cells in medium with MII-3H35 culture supernatant (3 μg/ml) for 48 hr (G), and 72 hr (H). × 100.

crease in the expression of the protein took place at the onset of myoblast fusion and treatment of MAb MII-3H35 inhibited myoblast fusion, we suggest that the protein plays a direct role in the fusion process. Rosenberg *et al.* (1985) reported a 105-kDa glycoprotein of similar behavior.

Monoclonal antibodies against myoblast have been used to distinguish mesenchymal, myogenic and chondrogenic precursors of developing chick embryo (Neff *et al.*, 1982; George-Weinstein, 1988) and a number of monoclonal antibodies have been isolated that define antigens whose ex-

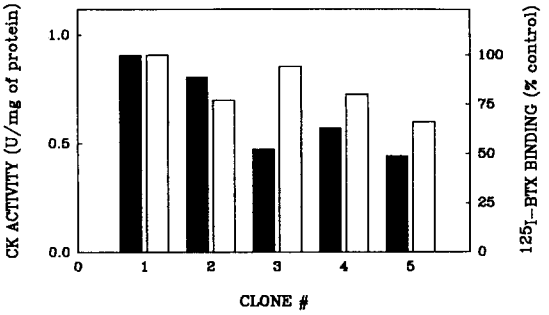

Fig. 3. Differential clonal effects on CK activity and acetylcholine receptor of cultured myoblast. Clone number 1, control; 2, MII-3; 3, MII-H13; 4, MII-3H18; 5, MII-3H35. ■, Creatine kinase activity; □, ^{125}I-bungarotoxin binding.

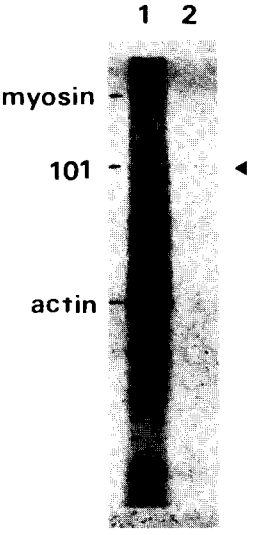

Fig. 4. Immunoprecipitation of the myoblast antigen by MAb MII-3H35. Cell extract of [^{35}S]methionine-labeled myoblast (lane 1) and immunoprecipitate with MAb MII-3H35 (lane 2) were analyzed by SDS-PAGE on 10% polyacrylamide gel under reducing condition.

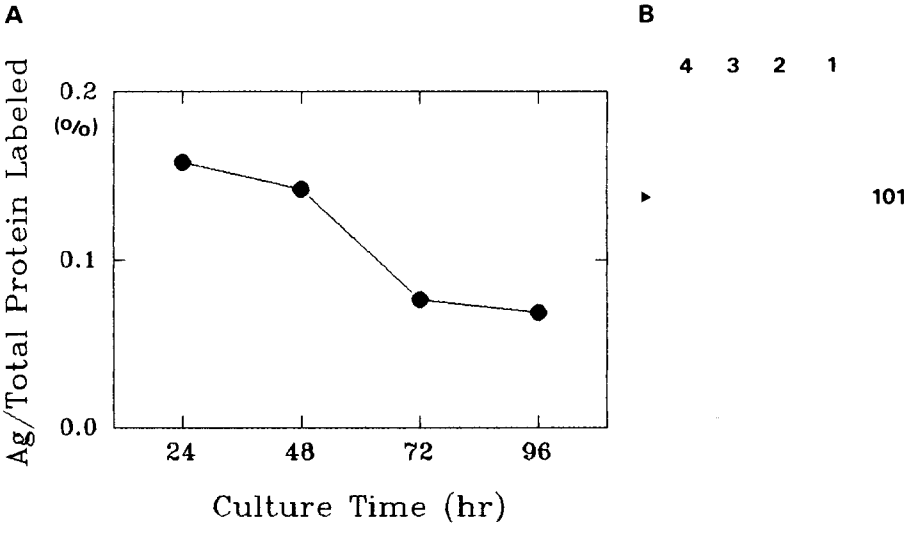

Fig. 5. Changes in the level of myoblast antigen of MAb MII-3H35 during the course of myogenesis in culture. A. Quantitation of antigens immunoprecipitated from [^{35}S]methionine-labeled myoblast with MAb MII-3H35. After separation of antigens by SDS-PAGE, the antigen bands were excised and the radioactivity was counted. B. Immunoblot analysis of antigens. Cultured myoblasts were immunoblotted with MAb MII-3H35 and detected with the anti-mouse-IgG conjugated with peroxidase. Myoblasts cultured for 24 hr (lane 1), 48 hr (lane 2), 72 hr (lane 3) and 96 hr (lane 4).

pression is decreased following myoblast fusion in skeletal muscle. These include the following antigens: Thy-1, 30.2A8, transferrin receptor, and 24.1D5 (Walsh *et al.*, 1984). The structural analysis, cell specificity and function of Ag MII-3H35 remains to elucidated.

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(Accepted November 30, 1991)

단일클론항체를 이용한 배양 계배 근원세포의 융합과 연관된 단백질의 확인

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배양 근원세포 분화에 따른 세포융합에 직접적으로 관여하는 단백질을 찾기 위해 48시간 배양한 근원세포를 항원으로 BALB/c mouse에 면역시켜 얻은 spleen cell과 myloma cell(P3U-1)을 융합시켜 근원세포와 반응하는 항체를 분비하는 hybridoma를 얻었다. 세 차례 한계 희석 후 계대배양하여 분리한 clone들이 분비하는 항체를 근원세포 배양액에 첨가하여 근원세포의 융합을 억제하는 효과가 있는 단일클론항체인 MII-3H13과 MII-3H18, MII-3H35를 얻었다. 이들은 농도의 증가에 따라 융합 억제 효과가 상승하는 농도의존성을 보여 3 μg/ml의 농도에서는 융합이 완전히 억제되었다. 이들 중 MII-3H35는 101-kDa의 단백질을 인지하였다. 이 101-kDa의 단백질은 근원세포 배양시 분화에 따라 그 양이 감소되어 융합 후에는 거의 나타나지 않았다. 따라서 101 kda의 단백질이 근원세포의 융합에 중요한 역할을 할 것으로 추정된다.