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Identification of tetranectin as adipogenic serum protein

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ABSTRACT

Fetal bovine serum (FBS) is an essential culture supplement for adipocyte differentiation of various adipogenic precursor cells. Adipocyte differentiation greatly varies depending on the type of serum in the differentiation medium. In this study, we found that FBS supported adipocyte differentiation of 3T3-L1 cells to a significantly higher extent than other types of bovine serum such as adult bovine serum (ABS). This differential adipogenic effect of bovine serum was shown to be due to the protein contents of bovine sera, indicating the presence of an adipogenic protein(s) in FBS. Serum proteome analysis identified tetranectin as an adipogenic protein. The adipogenic effect of tetranectin was confirmed by supplementation of FBS-containing differentiation medium with anti-tetranectin antibody, which suppressed adipocyte differentiation of 3T3-L1 cells. These results demonstrate that tetranectin is an adipogenic serum protein mediating the adipogenic effect of FBS.

1. Introduction

Adipocytes, predominant cells in adipose tissue, are formed by differentiation of various adipogenic precursor cells, which involves a complex regulatory network of intra- and extra-cellular molecules [1,2]. Adipocytes have the capacity of lipid accumulation, thereby contributing to energy homeostasis. However, an overabundance of adipocytes results in the development of obesity. It is well known that obesity is closely related to increased incidence of complex human diseases such as type II diabetes, cardiovascular disease and atherosclerosis [3,4]. Due to the growing obesity epidemic worldwide, study of adipocyte differentiation has become an intense research area in order to determine the underlying molecular mechanism for the treatment of obesity and related diseases.

A large part of the current knowledge concerning adipocyte differentiation has been clarified by studies of model cells, including primary cells and established cell lines [5,6]. The 3T3-L1 preadipocyte cell line derived from mouse embryos is most frequently used for the study of adipocyte differentiation [7]. Adipocyte differentiation of 3T3-L1 cells includes two distinct steps: proliferation of preadipocytes until confluence and growth arrest, followed by differentiation induced by hormonal stimulation with a defined adipogenic cocktail generally consisting of 3-isobutyl-1-methylxanthine, dexamethasone and insulin [5]. The proliferation of 3T3-L1 preadipocytes is carried out in culture medium generally containing calf serum (CS), which is replaced by fetal bovine serum (FBS) in adipocyte differentiation medium. FBS in differentiation medium was shown to support adipocyte differentiation to a significantly higher extent than other animal sera [8]. This suggests the existence of an adipogenic factor(s) in FBS that is absent or less prevalent in other animal sera. However, the superior adipogenic effect of FBS as compared to other animal sera has not been completely understood.

In this study, we compared the adipogenic effects of FBS and other types of bovine sera for adipocyte differentiation of 3T3-L1 cells. FBS in the differentiation medium supported adipogenic conversion of 3T3-L1 cells to a significantly higher extent than adult bovine serum (ABS). The adipogenic effect of FBS was significantly reduced by partial removal of FBS proteins but only slightly reduced by removing FBS lipids. In addition, supplementation of ABS-containing medium with a fraction of FBS proteins significantly increased the degree of adipogenic conversion of 3T3-L1 preadipocytes. These results indicate that the adipogenic effect of FBS was derived from serum proteins containing an adipogenic factor(s). Putative adipogenic proteins in FBS were screened by comparative serum proteome analysis of proteins fractionated from FBS and ABS. Tetranectin, a plasminogen kringle-4 binding protein, was identified as a putative adipogenic protein. Supplementation of FBS-containing medium with anti-tetranectin antibody significantly suppressed adipogenic conversion of 3T3-L1

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preadipocytes, confirming the adipogenic effect of tetranectin. Results of this study demonstrate that FBS contains an adipogenic protein(s) such as tetranectin involved in adipocyte differentiation of 3T3-L1 cells.

2. Materials and methods

2.1. Cell culture and differentiation of 3T3-L1 preadipocytes

Murine 3T3-L1 preadipocytes were maintained in DMEM (Hyclone) containing 10% calf serum (CS, Hyclone) by incubation at 37 °C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence and adipocyte differentiation was induced by exchanging the culture medium with differentiation medium (DMEM containing 10% fetal bovine serum (FBS, Hyclone) or 10% adult bovine serum (ABS, Hyclone) supplemented with induction cocktail (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 5 μg/ml of insulin (Sigma Aldrich)). After 2 days of differentiation induction, cells were further incubated in differentiation medium supplemented with 5 μg/ml of insulin for another 6 days by changing the medium every 3 days. In some experiments, differentiation media were supplemented with the indicated concentrations of FBSppt and/or anti-tetranectin antibody at every medium change. Adipocyte differentiation levels were determined by oil red-O staining measuring lipid contents in differentiated cells, as previously described.

2.2. Fractionation of serum proteins

Serum was fractionated into supernatant (FBSsup/ABSsup) and protein precipitate (FBSppt/ABSppt) by partial protein precipitation with 10% (w/v) polyethylene glycol 8000 (PEG), as previously described [9]. Protein precipitate was dissolved in PBS and protein concentrations were determined by Bradford assay [10]. Serum supernatant and the serum protein precipitate were filter-sterilized before addition into differentiation media.

2.3. Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent from 3T3-L1 cells in 5 days of differentiation. The cDNA was synthesized from 1 μg of RNA using a kit (Invitrogen) according to the manufacturer's protocol. Amplification reactions were conducted using an ABI 7500 Real-Time PCR system (Applied Biosystems) with the following primers for adipogenic marker genes: forward primer, 5′-AAGAGCTGACCCAATGGTTG-3′; reverse primer, 5′-ACCCTTGCATCCTTCACAAG-3′ for PPAR-γ, and forward primer, 5′-AGCCCAACTTGATCATCAGC-3′; reverse primer, 5′-ATGGTGGTC-GACTTTCCATC-3′ for FABP4.

2.4. Western blot analysis

Protein concentrations of bovine sera, FBSppt and ABSppt were determined by Bradford assay [10] and 50 μg each protein sample was subjected to 12% SDS-PAGE. Separated proteins were transferred to a PVDF membrane (Millipore). The membrane was blocked in 10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20 and 5% BSA (TBST buffer). Then, the membrane was reacted with anti-tetranectin antibody (1: 500, Santa Cruz Biotechnology) in TBST buffer overnight with constant agitation at 4 °C. After several washes with TBST, the membrane was incubated with peroxidase-conjugated anti-IgG antibody (Pierce). Proteins in the membrane were visualized using an enhanced chemiluminescence detection kit (Pierce) according to the manufacturer's instruction.

2.5. Statistical analysis

Data values were expressed as means ± SE. Differences in gene expression and intracellular lipid content were analyzed by an unpaired Student's t-test. A P value <0.05 was considered statistically significant.

3. Results

3.1. Adipogenic effect of FBS is significantly higher than that of ABS

Murine 3T3-L1 preadipocytes were differentiated by hormonal stimulation in medium containing fetal bovine serum (FBS) or adult bovine serum (ABS), as described in the materials and methods. Under a microscope, 3T3-L1 cells differentiated in FBS-containing medium showed the typical round-shape morphology of adipocytes along with accumulation of intracellular lipids droplets, indicating adipogenic conversion of 3T3-L1 cells (Fig. 1A). On the other hand, 3T3-L1 cells differentiated in ABS-containing medium mostly remained as preadipocytes, showing a bipolar fibroblast-like morphology without significant accumulation of intracellular lipid droplets. Determination of lipid contents in differentiated cells also showed that the degree of adipocyte differentiation was significantly higher in FBS-containing medium than in ABS-containing medium (Fig. 1B). In another experiment, 3T3-L1 cells were differentiated in medium containing a mixture of FBS and ABS at different ratios. The degree of adipocyte differentiation was determined to have proportionally increased with an increasing FBS:ABS ratio (Fig. 1B), again indicating that the adipogenic effect of FBS was significantly higher than that of ABS.

3.2. FBS lipids show a minor adipogenic effect

The superior adipogenic effect of FBS as compared to ABS indicates the presence of an adipogenic factor(s) in FBS, that is absent or less prevalent in ABS. We next examined the adipogenic effects of lipids and proteins, which are abundant serum components. To examine the adipogenic effect of lipids, lipid-depleted FBS was prepared by a previously developed method using organic solvents [11]. Delipidation of FBS did not significantly change either serum protein contents, as determined by SDS-PAGE analysis (Fig. 2A), or concentrations (~40 mg/ml), which were consistent with previous results [11]. 3T3-L1 cells were differentiated in medium containing lipid-depleted FBS, and the degree of adipocyte differentiation was determined by measuring intracellular lipid contents. The degree of adipocyte differentiation slightly decreased, but was insignificant (Fig. 2B). In another experiment, 3T3-L1 cells were differentiated in medium containing a mixture of delipidated FBS and ABS at different ratios. The degree of adipocyte differentiation was determined to have proportionally increased with an increasing the ratio of delipdated FBS:ABS (Fig. 2B). In addition, this increased level of adipocyte differentiation was similar to that obtained by increasing the ratio of lipid-containing FBS:ABS (Fig. 1B). These results indicate that the adipogenic effect of FBS was mostly retained in the lipid-depleted FBS.

3.3. FBS proteins contains an adipogenic factor(s) that mediates the adipogenic effect of FBS

To examine the adipogenic effects of proteins, FBS proteins were fractionated by partial protein precipitation using 10% (w/v) polyethylene glycol (PEG). Proteins precipitated from FBS (FBSppt) constituted ~3% of total FBS proteins, whereas proteins precipitated from ABS (ABSppt) constituted ~30% of total ABS proteins. The protein content of FBSppt was significantly different from that of

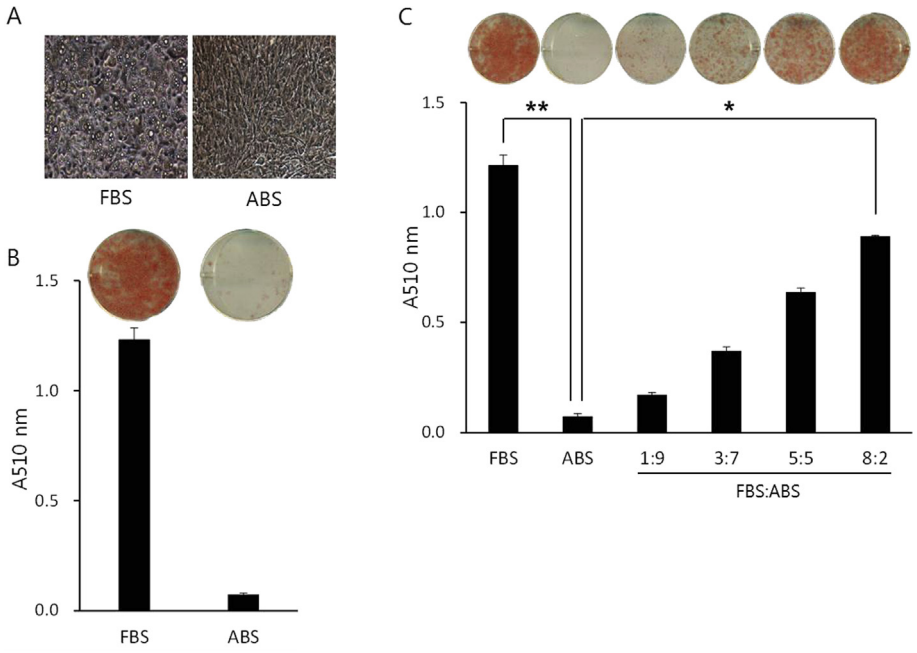

Fig. 1. Differential adipocyte differentiation depending of type of bovine serum in differentiation medium. 3T3-L1 cells were differentiated, as described in the materials and methods, in medium containing FBS, ABS or mixtures of FBS and ABS at the indicated ratios. Differentiated cells were observed under a microscope (A), and levels of adipocyte differentiation were determined by oil-red O staining (B and C). Data values in B and C are an average of n ≥ 5 and expressed as means ± SE: *, p value < 0.01; **, p value < 0.001.

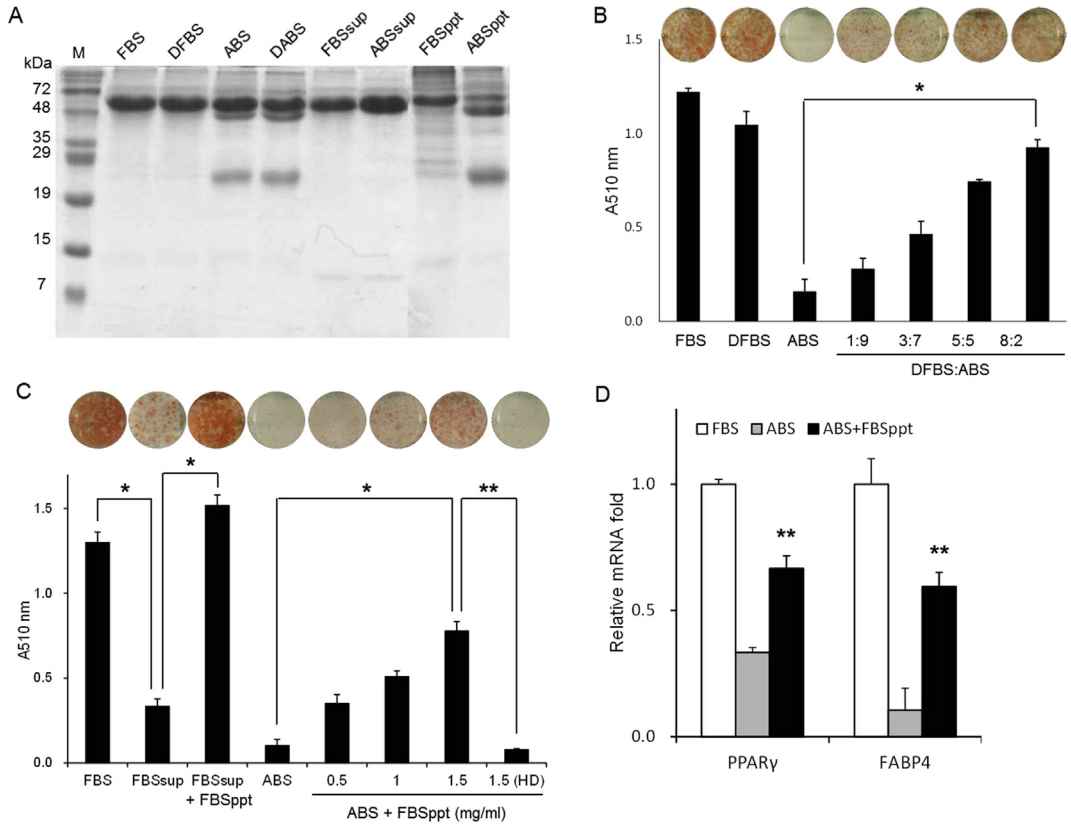

Fig. 2. Adipogenic effect of FBS derived from FBS proteins. SDS-PAGE analysis of proteins in FBS, ABS, delipidated sera (DFBS and DABS) and fractionated sera (supernatants (FBSsup and ABSsup), protein precipitates (FBSppt and ABSppt)) (A). The adipogenic effect of serum lipids was examined by differentiation of 3T3-L1 cells in medium containing FBS, DFBS or mixtures of ABS and DFBS at the indicated ratios (B). The adipogenic effect of serum proteins was examined by differentiation of 3T3-L1 cells in medium containing FBS, FBSsup, ABS, and supplemented with FBSppt or heat inactivated FBSppt (HD) at the indicated concentrations (C). Levels of adipocyte differentiation were determined by oil-red O staining (B and C) and quantitative real-time PCR (D). Data values in B, C, and D are an average of n ≥ 5 and expressed as means ± SE: *, p value < 0.01; **, p value < 0.001.

ABSppt, as shown by SDS-PAGE analysis (Fig. 2A). Protein amounts and contents of FBSppt and ABSppt did not change even when diluted sera were used for fractionation, indicating that the intrinsic properties of serum proteins determined protein precipitation from serum but not the protein concentration of serum. When 3T3-L1 cells were differentiated in medium containing FBSsup that remained after removal of FBSppt from FBS, the degree of adipocyte differentiation was significantly reduced to ~30% of that obtained in FBS-containing medium (Fig. 2C). Supplementation of FBSsup-containing medium with FBSppt resulted in recovery of adipocyte differentiation to that obtained in FBS-containing medium. In addition, supplementation of ABS-containing medium with FBSppt caused a dose-dependent increase in adipocyte differentiation (Fig. 2C). On the other hand, supplementation of ABS-containing medium with ABSppt did not cause any increase in adipocyte differentiation (data not shown). This adipogenic effect of FBSppt disappeared upon heat-denaturation of proteins in FBSppt. Consistently, expression levels of adipogenic marker genes (PPARγ and FABP4) significantly increased in cells differentiated in ABS-containing medium supplemented with FBSppt (Fig. 2D). These results indicate that the adipogenic effect of FBS was mediated by proteins mostly fractionated into FBSppt, which is absent or not prevalent in ABSppt.

3.4. Screening and identification of adipogenic proteins in FBS

Putative adipogenic proteins in FBSppt were screened by comparative 2D-PAGE analysis of FBSppt and ABSppt (Fig. 3A). Five protein spots (3008, 3009, 5007, 7007 and 7008) were specific for FBSppt (detected only in FBSppt but not in ABSppt). Two protein spots (4005 and 5002) were detected at >25-fold greater levels in FBSppt than in ABSppt. Peptide mass finger printing identified hemoglobin fetal subunit beta for FBSppt-specific protein spots and tetranectin for protein spots detected at greater levels in FBSppt than in ABSppt (Supplementary Table 1). Tetranectin was selected for further study (see discussion). Western blot analysis for tetranectin showed that the protein was significantly more abundant in FBSppt than in ABSppt (Fig. 4A), which is consistent with 2D-PAGE analysis. However, tetranectin was detected at similar levels in FBS and ABS. The adipogenic effect of tetranectin was examined by differentiation of 3T3-L1 cells in FBS-containing medium

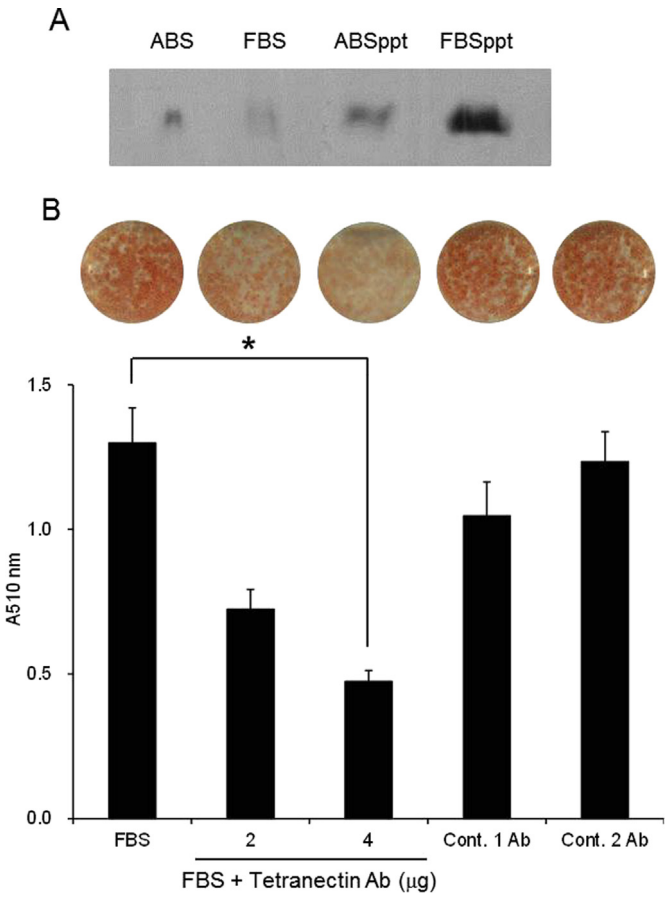

Fig. 4. Confirmation of adipogenic effect of tetranectin. Western blot analysis for tetranectin bovine sera, FBSppt and ABSppt (A). 3T3-L1 cells were differentiated, as described in the materials and methods, in medium containing FBS and supplemented with anti-tetranectin antibody or control antibodies (Cont. 1Ab, anti-Ig antibody; Cont. 2Ab, anti-glutaredoxin antibody). Degree of adipocyte differentiation was determined by oil-red O staining (B). Data values in B are an average of n ≥ 5 and expressed as means ± SE: *, p value < 0.01.

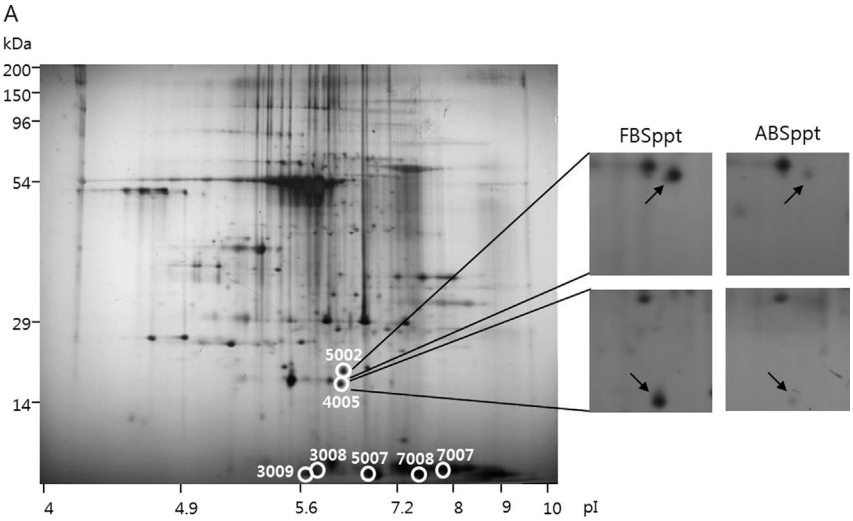

Fig. 3. Screening and identification of putative adipogenic protein(s) by 2D-PAGE analysis. Proteins in FBSppt and ABSppt were separated by 2D-PAGE and compared with each other (right panel), as described in the supplementary methods. FBSppt-specific proteins and proteins significantly more abundant in FBSppt than in ABSppt (right panels) were selected and further identified by peptide mass finger printing, as described in the supplementary methods.

supplemented with anti-tetranectin antibody. The degree of adipocyte differentiation was significantly suppressed in a dose-dependent manner by addition of anti-tetranectin antibody (Fig. 4B). However, other control antibodies for serum and intracellular proteins did not cause significant alteration of adipocyte differentiation. These results indicate that tetranectin is an adipogenic protein in FBS, and anti-tetranectin antibody inhibits the adipogenic effect of tetranectin to suppress adipocyte differentiation of 3T3-L1 cells.

4. Discussion

Animal cell culture media are generally supplemented with FBS, which is the most common type of bovine sera. FBS generally supports cell cultures more efficiently than other types of bovine sera, although the reason is not completely understood due to the biochemical complexity of serum components. Media for adipocyte differentiation of various adipogenic precursor cells are also supplemented with FBS, which has been shown to be efficient for adipocyte differentiation more than calf serum and other animal sera [8]. In this study, we showed that FBS is significantly more efficient than ABS for adipocyte differentiation of 3T3-L1 cells, which is consistent with previous results [8]. More importantly, we discovered that the adipogenic effect of FBS is mediated by proteins in serum, indicating that FBS proteins contain an adipogenic factor(s) for efficient adipocyte differentiation. Supplementation of ABS-containing medium with FBS proteins significantly enhanced adipocyte differentiation of 3T3-L1 cells (Fig. 2C), indicating that adipogenic proteins in FBS were not available or less prevalent in ABS. These results led us to identify an adipogenic protein(s) in FBS.

Albumin is a major serum protein comprising 50% of total serum protein content. Along with albumin, other high-abundance serum proteins such as immunoglobulin, transferrin, fibrinogen, haptoglobin and lipoproteins constitute >90% of total serum protein content [12]. High-abundance serum proteins are mostly common in animal sera, which is problematic for comparative serum proteome analyses that identify low-abundance proteins of interest. Several approaches have been developed to remove high-abundance proteins from serum prior to proteomic analyses, mainly based on antibody- or dye-based affinity depletion [13,14]. In this study, FBS proteins were fractionated into FBSppt and FBSsup, and most high-abundance proteins such as albumin (68 kDa) were detected in FBSsup (Fig. 2A). Proteins in FBSppt constituted ~3% of the total FBS protein content, whereas the adipogenic effect of FBS was detected in FBSppt (~70%) more than in FBSsup (~30%) (Fig. 2C). These results indicate that the adipogenic effect of FBS can be attributed to a low-abundance protein(s) in FBS that was efficiently fractionated into FBSppt. Fractionation of ABSppt from ABS also separated low-abundance proteins from most high-abundance proteins that remained in ABSsup. ABSppt constituted ~30% of the total ABS protein content, which showed an adipogenic effect that suppressed adipocyte differentiation of 3T3-L1 cells as previously reported [9]. Therefore, putative adipogenic proteins in FBS were screened by comparative 2D-PAGE analysis of proteins in FBSppt and ABSppt. Proteins showing specificity and abundance in FBSppt over ABSppt were selected as putative adipogenic proteins and identified by peptide mass finger printing.

Hemoglobin fetal subunit beta was identified as a putative adipogenic protein, which is a subunit of fetal hemoglobin comprising two alpha subunits and two fetal beta subunits [15]. Fetal hemoglobin is produced only in the fetus but is replaced by the adult form of hemoglobin comprising two alpha subunits and two adult beta subunits after birth. Hemoglobin is an oxygen-transport protein in red blood cells of all vertebrates. The level of extracellular free hemoglobin in blood circulation is low under normal conditions, although disease such as hemolytic anemia may show increased levels of serum hemoglobin. In this study, the fetal beta subunit of hemoglobin was detected as an FBSppt-specific protein, whereas the alpha subunit of fetal hemoglobin was not. This result indicates that ABSppt also contains free adult hemoglobin comprising alpha and adult beta subunits. Therefore, hemoglobin could unlikely mediate the adipogenic effect of FBS subunit and hemoglobin fetal beta subunit was excluded for further examination of its adipogenic effect.

Tetranectin, which was identified as the other putative adipogenic protein, is a homo-oligomeric protein found in serum as well as the extracellular matrix of certain human carcinomas [16]. Tetranectin has been shown to bind specifically to plasminogen kringle-4-domain [17], complex sulfated polysaccharides [18] and proteins in the extracellular matrix [19], indicating its role in processes involved in tissue remodeling. However, the physiological role of tetranectin has not been clearly elucidated. Recent studies have indicated that tetranectin plays an important role in osteogenesis and myogenesis [20–23]. In addition, tetranectin was shown to be involved in fat tissue development, according to age- and fat depot origin-dependent differential expression of the encoding gene in preadipocytes comprising fat tissues [24]. In this study, we demonstrated that tetranectin in FBS plays an important role in adipocyte differentiation of 3T3-L1 cells, which was efficiently suppressed by binding to anti-tetranectin antibody (Fig. 4B). Although tetranectin was significantly more abundant in FBSppt than ABSppt, its protein level was apparently similar in both ABS and FBS. This observation suggests that other serum protein(s) or non-protein factor(s) regulate the function of tetranectin, which mediates the adipogenic effect of serum. In fact, ABS was shown to contain an anti-adipogenic protein(s) that suppresses adipocyte differentiation of 3T3-L1 cells, which might inhibit the adipogenic function of tetranectin [9]. In conclusion, proteins in FBS contain an adipogenic factor(s) that mediates the adipogenic effect of the serum. Serum proteome analysis identified tetranectin as adipogenic protein. The adipogenic effect of tetranectin was demonstrated by using anti-tetranectin antibody, which suppresses adipocyte differentiation in FBS-containing medium. Further investigations will clarify the adipogenic function of tetranectin, which is an ongoing topic of study in our group.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.073.

Transparency document

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