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REVIEW

Characterization of PSPA Cell Differentiation, an Established Pig Preadipocyte Cell Line as an In Vitro Model for Pig Fat Development

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Abstract

We have established a clonal porcine subcutaneous preadipocyte cell line for the first time. This cell line, named PSPA, shows a fibroblast-like phenotype and keeps on growing under growth medium even after reaching confluence. Adipose conversion occurs only when confluent cultures are stimulated with insulin, dexamethasone, biotin, pantothenate, and octanoate. Following exposure to this differentiation medium, growth was arrested immediately without mitotic expansion and PSPA cells exhibited a marked increase in intracellular lipid accumulation accompanied by changes in gene expressions directly related to fat metabolism and also in extracellular environments. Since PSPA cells were established, it has become possible to end the discussion over whether there is species specificity in the adipose conversion between pig and mouse by comparing PSPA cells and 3T3-L1 cells, the most widely studied preadipocyte cell line. Consequently, we demonstrated that responsiveness to inducers and fatty acid composition was unequal, despite being treated with the same media. Thus, species-specific differences indeed exist in adipogenesis between these two, meaning that PSPA cells would be an in vitro model specialized to improve understanding of pig adipocyte growth and differentiation in controlling the carcass fat content of pigs.

Discipline: Biotechnology
Additional key words: 3T3-L1, extracellular matrix, fatty acid, pig backfat, subcutaneous adipose tissue

Introduction

Subcutaneous adipose tissue in pigs represents a major source of both cost inefficiency and consumer concerns. Numerous investigations have been performed to regulate the carcass fat for meat quality traits through both nutritional controls and selective breeding (Wood et al. 1999, de Vries et al. 2000, Bonneau & Lebret 2010). However, there are some difficulties in monitoring adipose development in in vivo studies, due to the highly complex interactions of all kinds of neural and hormonal signals involved in the adipocyte metabolism. Therefore, previous investigators have established in vitro cell culture systems as useful tools for studying adipose development. Much of the knowledge about cellular and molecular events accompanying adipose conversion in recent years has been based on preadipocyte cell lines originating from rodents (Green & Kehinde 1975, Négrel et al. 1978). Since no cell lines from porcine adipose tissue have been established to date, understanding how adipocyte differentiation is controlled in pigs is limited to either an approach of using these mouse cell lines or otherwise, primary culture systems for short life usage (Hentages & Hausman 1989, Suryawan & Hu 1993, Gelfault et al. 1999). As a result, the question always remains: Do the data from mouse cell lines and pig primary stromal-vascular cells with cellular heterogeneity in its population really represent the characteristics of pig adipocytes? Indeed, species specificity often becomes a point of debate, particularly when primary cultures behave somewhat differently from established mouse cell lines. Thus, based on these problems, providing specific culture systems of pig preadipose cells without containing other cell types and which can be passaged indefinitely as well as cell lines is inevitable to clearly understand the mechanisms of pig fat development.

We have established a porcine preadipocyte cell line (PSPA) as an in vitro model system which clonally derived from subcutaneous tissue (Nakajima et al. 2003). Here, in

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this review, PSPA cell profiles will be introduced and we also discuss how the PSPA cell is suitable for the pig fat study.

Establishing the porcine preadipocyte clonal cell line, PSPA

1. Isolation and cloning of porcine preadipocytes

Porcine preadipocytes were obtained according to Forest et al. (1987). Dorsal subcutaneous tissue was dissected from crossbred fetuses (27, 28, 58, 85, 93 days gestation) and newborns, though only that from 85-day fetuses was successful in obtaining the cell line. Tissue samples were minced and digested in Dulbecco's modified Eagle's medium (DMEM, 1 g/L glucose) containing 1 mg/mL collagenase for 30 min at 37°C. After filtration through a 75-μm mesh and centrifugation, the supernatant was discarded. The pellet fraction, primarily comprising of stromal-vascular cells, was resuspended and seeded into a tissue culture flask in the growth medium, DMEM supplemented with 10% fetal bovine serum (FBS). The cell monolayer was tripsinized, and cells were then cloned by a limiting dilution. Two to four weeks later, the different clones were grown separately, and the PSPA clone displaying the highest frequency of adipose conversion was selected.

2. PSPA cell culture

Based on the growth curve study, we decided it was suitable to pass the cells every 3~4 days at a constant density of inoculation of 1×10^4 cells/cm^2 (Fig. 1). Exponentially grown PSPA cells exhibited a fibroblastic appearance (Fig. 2a). PSPA cells have undergone at least 85 passages with no detectable loss of phenotypic properties. Under non-adipogenic conditions in growth medium, they never spontaneously differentiate into adipocytes (Fig. 2b).

In order to produce adipocytes, PSPA cells were plated at 2.1×10^4 cells/cm^2 and grown for 3 days to obtain confluency. After reaching confluence (day 0), adipose conversion was induced by various combinations of hormonal factors (high glucose (4.5 g/L) DMEM containing 10% FBS in addition to 5 μg/mL insulin, 0.25 μM dexamethasone, 33 μM biotin, 17 μM pantothenate, and 5 mM octanoate) to determine the optimum adipogenic medium for the induc-

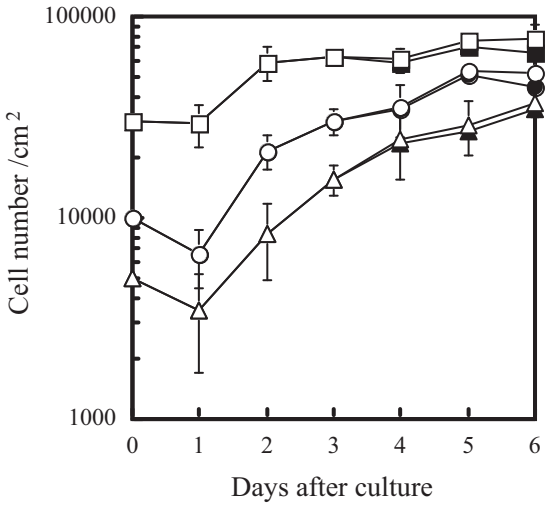

Fig. 1. Growth curves of PSPA cell
Cells were inoculated at three different starting densities, 0.5×10^4 (△), 1×10^4 (○) and 3×10^4 cells/cm^2 (□), and cell numbers were determined from the following day using a hemocytometer. Closed symbols show cell viability resulting from the trypan blue exclusion assay. Values are the mean ± SD of four determinants.

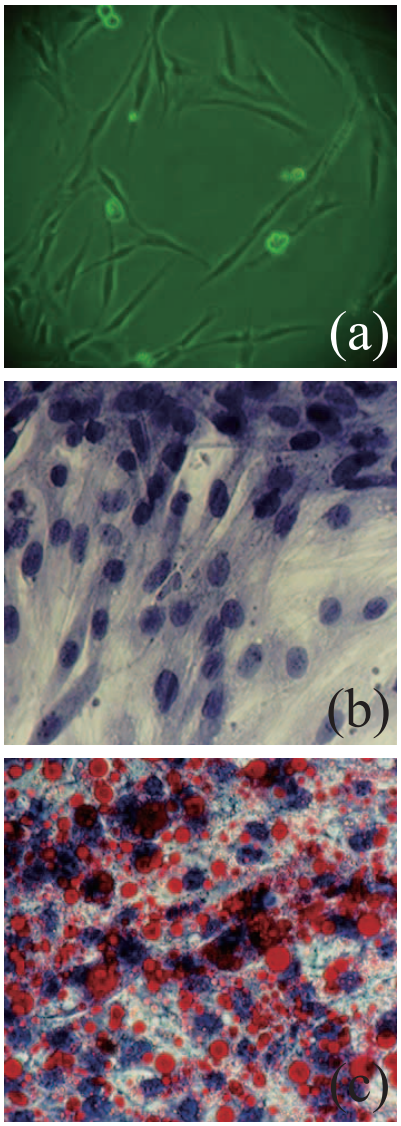

Fig. 2. Photomicrographs of the PSPA cell
(a) Exponentially growing PSPA cells with fibroblastic morphology. x100. (b, c) Oil-red-O staining of PSPA cells 10 days after confluency cultured under either growth medium (b) or differentiation medium (c). x400.

tion of PSPA adipocytes. The medium was changed every other day and the cells were allowed to differentiate for a further 10 days. The triglyceride (TG) accumulation was markedly accelerated by treating the cells with medium containing all the agents (Fig. 3). Thus, this mixture was used in the differentiation medium of PSPA cells. Oil-red-O staining of 10 days' culture after stimulation allows adipocytes occupied by numerous intracellular lipid droplets to be observed (Fig. 2c). PSPA cells retain their ability to develop and differentiate into mature adipocytes after more than 60 passages.

Changes in PSPA cells from fibroblast-like preadipocytes to fat-laden adipocytes

1. Intracellular changes during adipose conversion

The typical hallmark of the adipogenesis process is the de novo synthesis of numerous and large cytoplasmic lipids. Many of the changes that occur during adipose conversion take place at the gene expression level. Several reviews have also summarized the genes differentially regulated during adipogenic program in early, mid-, and late markers (Ailhaud et al. 1992, Smas & Sul 1995, Gregoire 2001). As we experimented using the RT-PCR technique in Fig. 4, only stimulated PSPA adipocytes demonstrated adipose-specific characteristics, including those master regulators of adipocyte gene transcription (PPARγ2 and C/EBPα), and further enzymes needed to implement de novo lipogenesis (LPL, aP2, SCD1, and HSL). On the other hand, as often applies to some other differentiated cell types, withdrawal from the cell cycle precedes adipocyte differentiation. Committed PSPA cells, which have ceased dividing, down-regulated genes associated with cell growth (E2F1, PCNA, and TK), whereas those highly expressed in proliferative undifferentiated preadipocytes lacking a series of molecular markers of mature adipocytes.

2. Extracellular changes during adipose conversion

Besides dramatic increases in gene expression and proteins directly related to lipid metabolism, alterations also occur in the extracellular environment during adipocyte differentiation. Ultrastructural studies have shown that each adipocyte is supported by a basement membrane and is interconnected with abundant fibers and rods which cannot be seen in fibroblast-like preadipocytes (Napolitano 1963, Kuri-Harcuch et al. 1984). These newly accumulated extracellular matrix (ECM) macromolecules are comprised of various numbers of collagens, structural glycoproteins and proteoglycans. As previously reported in both mouse and bovine adipocytes (Kubo et al. 2000, Nakajima et al. 2002a), we also observed the same phenomenon with porcine adipocytes in which a prominent fibrillar network of collagens developed only in differentiated cells, thus forming fat cell

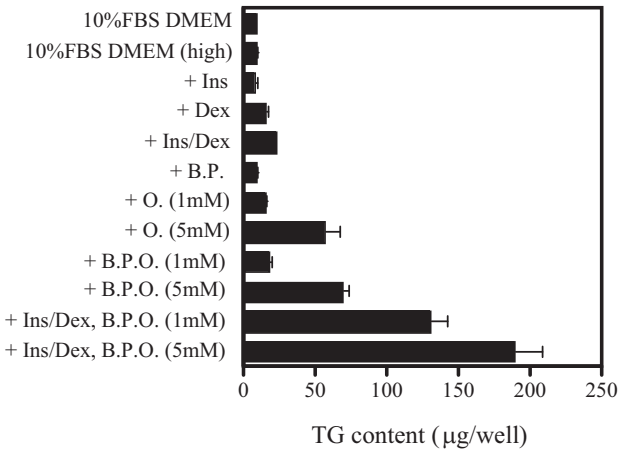

Fig. 3. Stimulating PSPA cell with various adipogenic factors

Confluent PSPA cells were exposed to various combinations of inducing factors and their TG contents were measured at day 10. Values are the mean ± SD of three determinants. High, high glucose DMEM; Ins, insulin; Dex, dexamethasone; B., biotin; P., pantothenate; O., octanoate.

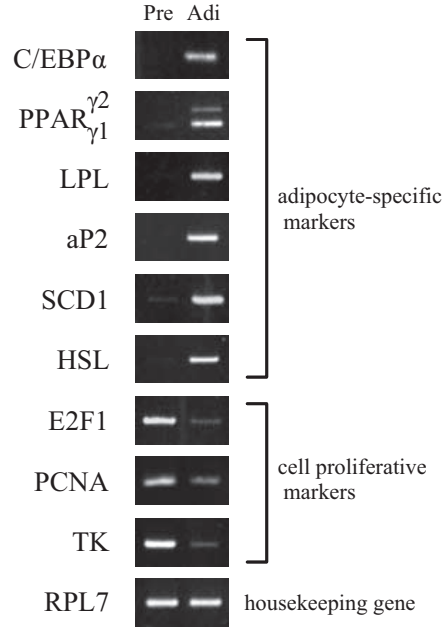

Fig. 4. Gene expressions between PSPA preadipocytes and adipocytes

RT-PCR experiments were performed on mRNAs extracted from PSPA preadipocytes maintained in growth medium and PSPA adipocytes exposed to differentiation medium on day 10 after confluence. Pre, preadipocyte; Adi, adipocyte, C/EBP, CCAAT enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; LPL, lipoprotein lipase; aP2, adipocyte fatty acid binding protein; SCD, stearoyl CoA desaturase; HSL, hormone-sensitive lipase; E2F, adenovirus E2 promoter-binding factor; PCNA, proliferating cell nuclear antigen; TK, thymidine kinase; RPL7, ribosomal protein L7.

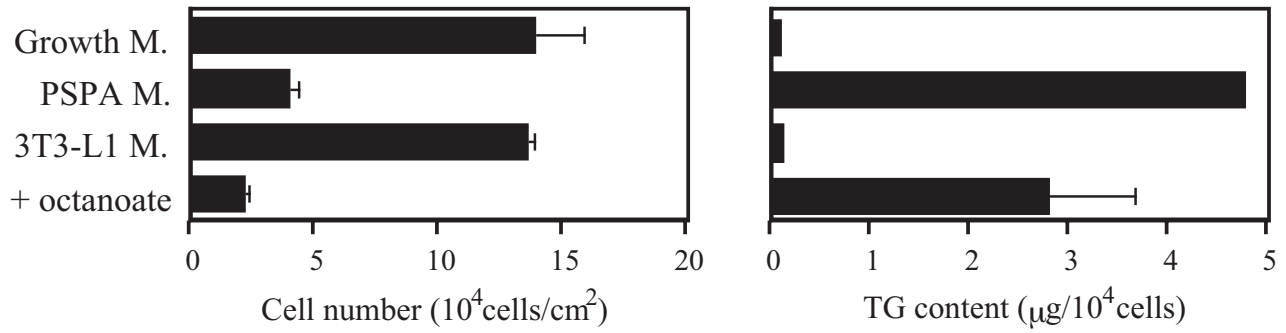

Fig. 5. Effect of differentiation medium on cell growth and TG accumulation of PSPA cells
Confluent PSPA cells were treated with the following four media: growth medium (Growth M.), PSPA differentiation medium (PSPA M.), 3T3-L1 differentiation medium (3T3-L1 M.), and 3T3-L1 medium supplemented with 5 mM octanoate (+octanoate). After 10-day culture, cell numbers and TG contents were measured. Values are mean ± SD of four determinants.

clusters (unpublished data). In addition, impairing these ECM networks by treatment of ethyl-3,4-dihydroxybenzoate, a specific inhibitor of collagen synthesis, reduced the level of adipogenesis in mice and bovine (Ibrahimi et al. 1992, Nakajima et al. 2002b), as well as pig adipocytes (unpublished data). Although it is unclear why adipose cells become interconnected with collagen fiber networks, these collagens seem essential across the animal species to accomplish the terminal differentiation program of adipocytes.

Caution against using mouse culture model for pig fat studies

1. Species-specific difference in responsiveness to differentiation medium

The most extensively characterized and widely studied cell models for preadipocyte differentiation are the murine 3T3-L1 cells. By comparing this most frequently studied mouse cell line and PSPA cells, we demonstrated that there are some species specificity in the mode of adipocyte differentiation between mice and pigs. First, PSPA cells did not show the so-called clonal expansion phase to enter terminal differentiation like 3T3-L1 cells as reported by Gregoire (2001). Second, the most striking evidence was that the standard hormonal cocktail of 3T3-L1 cells (0.5 mM 1-methyl-3-isobutylxantine, 0.25 μM dexamethasone, 5 μg/mL insulin) failed to induce PSPA cells to differentiate, but kept on growing, even after confluence, as did the preadipocytes in growth medium (Fig. 5). Similar results were obtained using porcine primary culture isolated from 5-mo-old crossbred pig and another Chinese breed (unpublished data).

By serially omitting each individual adipogenic agent from the PSPA differentiation medium, octanoate and dexamethasone were determined as the key factors for porcine preadipocytes to differentiate. Moreover, octanoate was the only factor capable of inducing growth arrest. Octanoate supplementation to 3T3-L1 medium increased the TG accumulation of PSPA cells accompanied by growth arrest (Fig. 5). On the other hand, when another essential factor, dexamethasone, was depleted from the PSPA medium, the cell numbers were maintained as normal adipocytes, thereby indicating that dexamethasone regulates PSPA differentiation through a different induction pathway from octanoate. Finally, RT-PCR and Western blot analysis supported the idea of octanoate and dexamethasone as requisites for porcine preadipocytes to enter terminal differentiation by affecting the expression of those master regulators of adipocyte gene transcription factors, C/EBPα and PPARγ2, respectively. (Nakajima et al. 2003).

Growth arrest by octanoate was effective for PSPA cells to enter terminal differentiation. These results imply why earlier researchers often preferred serum-free conditions in porcine primary cultures instead of serum-containing medium (Hentges & Hausman 1989, Suryawan & Hu 1993, Gerfault et al. 1999). This was because none of the agents added according to mouse reports were sufficient to make preadipocytes cease dividing, whereas it was easily achieved by serum-free condition.

2. Species-specific difference in fatty acid composition of adipocytes

Species specificity not only existed in the responsiveness to inducers upon adipose conversion, but was also found in fatty acid components of cellular TG between pig and mouse cell lines. Table 1 summarizes the percentages of major fatty acid components of cell lipid extracts from PSPA cells and 3T3-L1 cells of 10-day culture determined by gas chromatography. The lipid accumulated adipocytes of both cell lines increased in proportion of C16:1, which was poorly detected from fibroblastic preadipocytes maintained in growth medium and PSPA cells in 3T3-L1 differ-

Table 1. Fatty acid composition of PSPA and 3T3-L1 (%)

	PSPA			3T3-L1		
Acyl chains	Growth M.	PSPA M.	3T3-L1 M.	Growth M.	PSPA M.	3T3-L1 M.
8:0	0	20.9	0	0	20.1	0.1
10:0	0	1.7	0	0	0.9	0.1
12:0	0	0.7	0.9	0	0.4	0.2
14:0	0	3.4	0	0	2.0	3.5
14:1	0	0.8	0	0	0.4	1.3
15:0	0	0	0	0	0.6	4.9
15:1	0	0	0	0	0	1.4
16:0	25.9	11.2	24.4	27.2	23.0	30.8
16:1	0	17.2	1.9	1.9	13.8	40.7
17:0	0	0.2	0	0	0.7	1.1
17:1	0	0.4	0	0	1.0	4.1
18:0	17.5	4.6	13.8	16.1	5.9	1.0
18:1 n-9	23.0	25.6	27.2	5.6	22.9	8.0
20:4	10.2	1.5	8.6	14.2	1.4	0.1

Fatty acid composition analysis was performed on a Shimadzu GC-17A gas chromatograph with a Shimadzu capillary column (HSS1-PM30) at an initial oven temperature of 150°C, final temperature of 220°C, heating rate of 3°C/min, injector temperature of 250°C and detector temperature of 250°C. The carrier gas (He) was supplied at 80 kPa, hydrogen gas at 60 kPa and compressed air at 50 kPa. The sample was injected with splitless mode. Cellular TG was extracted with chloroform-methanol (2:1, v/v) 10 days after being cultured with either growth medium (Growth M.) or PSPA differentiation medium (PSPA M.) or 3T3-L1 differentiation medium (3T3-L1 M.). Subsequently, fatty acids were esterified using sodium methylate (0.5 N). Results are shown as percentages of means from three independent experiments.

entiation medium. For cells treated with PSPA differentiation medium, 20% of acyl chains of TG were composed of C8:0 in both pig and mouse adipocytes, indicating that a considerable amount of octanoate was simply incorporated from the medium. On the other hand, it was of interest that each cell line seemed to have its own fatty acid profiles regardless of all tested culture media; always with a higher abundance of saturated and monounsaturated C18 than C16 fatty acids in PSPA cells, whereas saturated and monounsaturated C16 rather than C18 fatty acids predominated in 3T3-L1 cells. These results resembled the data from fatty acid components of pig fat and rat fat (Christie et al. 1991, MEXT), thus indicating these cellular models conserve the nature of their original species. In addition, a significant amount of odd chain fatty acids, such as C15 and C17, identified especially in 3T3-L1 TG, also agreed with former reports (Glenn et al. 1992). Taken together, we propose that mouse is an unfit model system, at least in part, for the study of porcine adipose development.

Future perspectives

What is the advantage in using PSPA cells? For example, an established cell line will greatly facilitate the study of the molecular details of adipose conversion at the transcriptional level from comparing the adipose state to the preadipose state (Matsumoto et al. 2012). In addition, the role of some candidate genes in relation to the pig fat development will also make it possible to demonstrate by using PSPA cells with gene transfer technique (Taniguchi et al. 2014). Further, this simple culture system allows us to readily monitor adipose development under conditions of controlled supplements and substrates to cultural medium easily to determine the effect of some diets for the possibility to regulate pig fat (Suzuki et al. 2008, Irie et al. 2012). However, over the past two decades, the view of adipose tissue has undergone a dramatic change from an inert excess energy reservoir to a dynamic endocrine organ (Wang et al. 2008, Deng & Scherer 2010). Nevertheless, it is certain that, together with the usual animal experiments and recently reported pig genome databases (Martien et al. 2012), the PSPA cell culture model will become even more helpful to further our understanding of the detailed mechanisms of pig fat development.

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