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Cell Density-Dependent Transcriptional Activation of Endocrine-Related Genes in Human Adipose Tissue-Derived Stem Cells

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Abstract

Adipose tissue is recognized as an endocrine organ that plays an important role in human diseases such as type II diabetes and cancer. Human adipose tissue-derived stem cells (ASCs), a distinct cell population in adipose tissue, are capable of differentiating into multiple lineages including adipogenesis. When cultured *in vitro* under a confluent condition, ASCs reach a commitment stage for adipogenesis, which can be further induced into terminally differentiated adipocytes by a cocktail of adipogenic factors. Here we report that the confluent state of ASCs triggers transcriptional activation cascades for genes that are responsible for the endocrine function of adipose tissue. These include insulin-like growth factor 1 (IGF-1) and aromatase (Cyp19), a key enzyme in estrogen biosynthesis. Despite similar adipogenic potentials, ASCs from different individuals display huge variations in activation of these endocrine-related genes. Bioinformatics and experimental data suggest that transcription factor Foxo1 controls a large number of “early” confluency-response genes, which subsequently induce “late” response genes. Furthermore, siRNA-mediated knockdown of Foxo1 substantially compromises the ability of committed ASCs to stimulate tumor cell migration *in vitro*. Thus, our work suggests that cell density is an important determinant of the endocrine potential of ASCs.

Keywords

adipose stem cells; cell confluency; aromatase; gene expression profiling; SBSN; Foxo1; adipogenesis commitment

INTRODUCTION

Obesity, which has reached an epidemic scale in this country, is associated with various pathological conditions such as type II diabetes, cardiovascular diseases, and certain cancers

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[1,2]. A wealth of evidence strongly suggests that adipose tissue is a highly active endocrine organ that secretes numerous factors, including growth factors, cytokines, hormone-like molecules, complement-related proteins, and extracellular matrix (ECM) proteins [3-7]. Excessive production of many of these factors by adipose tissue at least partially accounts for obesity-associated disease risk. For example, obesity is a well-known risk factor for postmenopausal breast cancer [8-10]. One of the probable causes is the increased expression of aromatase, a key enzyme involved in estrogen biosynthesis, and elevated local estrogen production in adipose tissue of postmenopausal obese women [11,12]. In addition, adipokines and cytokines produced by adipose tissue may also contribute to breast cancer risk [13]. For example, leptin has been implicated as a growth factor in mammary tumor development [14, 15]. Insulin-like growth factor 1 (IGF-1) is another tumor-promoting factor whose production is positively correlated with breast cancer risk [16-18].

While fully differentiated, fat-laden adipocytes are the major source for several extensively characterized adipokines such as leptin and adiponectin [18], other resident cell types in adipose tissue could also contribute to obesity-associated chronic inflammation. For example, macrophages in adipose tissue produce a number of inflammatory factors such as TNF- α , IL-6, and MCP-1 [19-21]. In addition, precursors of adipocytes are far more potent in aromatase expression and estrogen biosynthesis than adipocytes themselves [22,23]. Although adipocytes have been extensively investigated for their roles in cancer initiation and progression, little attention has been given to the potential of their precursors in the disease development and progression.

It is well accepted that obesity is caused by increases in both size (hypertrophy) and number (hyperplasia) of adipocytes [24-27]. The steady-state number of adipocytes is a net result of differentiation of progenitor cells and apoptosis of adipocytes. Fat progenitor cells, which were recently identified from the stromal-vasculature compartment of mouse adipose tissue [27, 28], can be directed toward proliferation and adipocyte differentiation by an intricate network of nutritional, hormonal, and paracrine signals [29]. Human counterparts of mouse fat progenitor cells, termed adipose tissue-derived stem cells (ASCs), have also been isolated and characterized [30-32]. ASCs display extensive developmental plasticity, as evidenced by their ability to differentiate into multiple cell types including adipocytes, osteoblasts, chondrocytes, and myocytes, etc. The relative abundance and expendability of ASCs have raised high enthusiasm over their regenerative potentials. However, recent studies from others and ours indicate that ASCs are capable of secreting various tumor-promoting factors including IL-6 [33-35]. In addition, spontaneous transformation of ASCs after prolonged *in vitro* incubation also raises safety concerns over their therapeutic potential in regenerative medicine [32,36].

Adipogenesis can be divided into two stages: adipocyte commitment of multipotent stem cells and terminal differentiation to mature adipocytes [37,38]. Adipocyte commitment under tissue culture conditions is achieved by cell confluency. Cells under high confluency can be further induced to become mature adipocytes by various adipogenic inducers. Transcriptional pathways important for the later stages of adipogenesis have been extensively studied [24-26,38]. In particular, several key transcription factors such as PPAR γ and members of the C/EBP protein family (e.g. C/EBP α and C/EBP β) play pivotal roles in dictating cascades of transcriptional activation required for establishing and/or maintaining the terminally differentiated state [39,40]. In contrast to the understanding of the molecular control of the final steps of adipogenesis, limited knowledge is available regarding the molecular events that take place during the commitment stage. Recent studies of mouse preadipocyte cell lines revealed that pro-adipogenic factor such as bone morphogenetic protein-4 (BMP-4) [41] and antiadipogenic factors such as Pref-1 and Nurr1 [42,43] play critical roles in the commitment stage of adipogenesis. However, a comprehensive picture of the transcriptional response to signals for adipogenic commitment is still lacking.

In the current study we carried out a gene expression profiling study of ASCs cultured under the confluent condition. We found that the density-driven cellular mechanism induced distinct waves of transcriptional activation of many known inflammatory and endocrine-related genes. Our work also indicates that transcription factor Foxo1 serves as a key regulator for the expression of numerous growth factors and ECM proteins in ASCs.

MATERIALS AND METHODS

Cell culture and treatment

Primary human ASCs were isolated from healthy patients undergoing elective cosmetic surgical procedures at the University of Virginia, using methods previously published [30] and approved by the University of Virginia's Human Investigation Committee. Patient consent was acquired prior to tissue procurement. As fat tissue would have been otherwise discarded, the procedure did not deviate from the standard care, and it posed minimal risk to the donors. Fat tissue was procured by excision or liposuction, washed thoroughly, and enzymatically digested. Digested tissue was filtered to remove debris and stromal cells were pelleted by centrifugation. Contaminating erythrocytes in the stromal pellet were cleared up by incubation with an osmotic erythrocyte lysis buffer and subsequent centrifugation [30]. ASCs were further enriched by culturing on a plastic surface in DMEM/F-12 medium with 10% fetal bovine serum (FBS) and antibiotic-antimycotic solution, using previously described methods [44]. Sub-confluent and confluent ASC cultures were plated at 2×10^5 and 1.1×10^6 per 10 cm tissue culture dish, respectively. A cohort of 11 ASC samples were used in Supp. Fig. 1.

Microarray and data analysis

ASCs were cultured under sub-confluent and confluent conditions in regular growth medium. Total RNA was harvested from two biological replicates of sub-confluent, 2d, and 12d confluent cultures, and was subjected to microarray analyses using Affymetrix HG-U133A GeneChips™ as described earlier [45]. Variations in gene expression between sub-confluent and confluent samples were considered significant if the p-values were equal to or less than 0.05, the fold changes were equal to or greater than 2, and the absolute difference in signal intensity was greater than 100 (approximately 3 times of noise level, defined by 20% of mean intensity of all absent probe sets). dChip software package was used to analyze the microarray data [46]. For the Present or Absent Calls, p-values were computed by a non-parametric Wilcoxon's rank test comparing the population of PM probes and MM probes for each gene on the chip. Gene Ontology analysis was performed using DAVID software from the National Institutes of Health (<http://david.abcc.ncifcrf.gov>).

To search for over-represented transcription factor (TF)-binding sites, the affymetrix IDs of up-regulated genes were first converted to official gene symbols using DAVID (<http://david.abcc.ncifcrf.gov>). The gene symbols were then input into Whole Genome rVista [47] for discovery of conserved and over-represented TF binding sites occurring in the 1kb promoter sequence upstream of the transcription starting site of a gene in both human and mouse genomes. The motifs found by Vista are known transcription factor binding sites from the TRANSFAC database [46], and are conserved between human (March 2006 assembly) and mouse (February 2006 assembly) [47]. The significance of enrichment for a motif in a set of genes is determined by a p-value based on the number of occurrences of the motif in the 1kb promoter regions of this set of genes as compared to the total number of occurrences of the motif in all promoter regions of human RefSeq genes, and adjusted for multiple-hypothesis testing [47].

RNA isolation, cDNA synthesis, and quantitative real-time RT-PCR

Total RNA was extracted with TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Quality of RNA samples was verified by absorbance measurements at 260 and 280 nm. To prepare cDNA, approximately 1 µg of RNA was reverse-transcribed using ImPromp (Promega, Madison, WI, USA).

Results obtained from the Affymetrix microarray were verified by real-time RT-PCR using gene-specific primers (Supp. Table 3). Primers used for BRCA1 [48], rRNA (48), aromatase [48, 49], and WISP2 [50] were previously reported. Primer Express software (Applied Biosystems, Foster City, CA, USA) was used to design primers that cover two neighboring exons. SYBR Green technology and an ABI7900 real-time PCR machine were used for the quantitative real-time PCR analysis. Expression levels were normalized against 18S rRNA.

Immunoblotting and antibodies

An equal amount of whole-cell lysates was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were detected by anti-Foxo1 (Santa Cruz Biotech., Santa Cruz, CA, USA) and anti- α -tubulin (Calbiochem, San Diego, CA, USA) antibodies.

siRNA knockdown

Gene-specific knockdown by siRNA oligonucleotides was conducted using Lipofectamine reagent RNAiMAX (Invitrogen). The knockdown experiment was performed as previously described [48]. Briefly, cells at a density of 60% were first transfected with siRNA oligos at a final concentration of 10 nM. For the comparison of sub-confluent and confluent cells, cells were trypsinized 24 hr after transfection and plated in sub-confluent or confluent densities. Cells were harvested for RNA analysis 72 hr after plating. Foxo1 (M-003006-01-0005), SBSN (M-027259-00-0005), ACTC1 (M-012015-00-0005), and control (D-001810-10) siRNA oligos were purchased from Dharmacon, Inc. (Lafayette, CO).

Adipogenesis

For the adipogenesis assay, ASCs were grown until they reached confluency in regular medium. They were then switched to DMEM/F-12 containing 1 µM dexamethasone (Sigma), 10 µM insulin (Sigma), 200 µM indomethacin (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), for further growth for 10 days. ASCs following adipogenesis were stained with Oil-Red-O, together with the control cells growing under regular medium.

Boyden-chamber migration assay

4×10^4 siRNA-treated ASCs were seeded in a 24-well plate (cat. #353504, BD Biosciences, Bedford, MA) and overlaid with 0.5 ml DMEM/F12 plus FBS followed by an incubation for 48 hr at 37°C. The migration assay was carried out by placing 5×10^4 MDA-MB-231 breast cancer cells in the upper compartment of the Boyden-chamber system, using trans-well inserts fitted with 3 micron pore membranes (BD Biocoat cat. # 354575). The assay was carried out for 7 hr, after which the inserts were removed and the cells subsequently fixed in 4% paraformaldehyde. The cells were then stained with a 0.1% Crystal-Violet solution. Tumor cells on the upper membrane surface were removed with a paper towel. The air-dried membranes were viewed under 20x magnification and migrated cells were counted in 3 randomly chosen fields per membrane. The assay was carried out in triplicate and repeated twice.

RESULTS

Whole genome analysis of confluency-triggered gene expression changes in ASCs

To determine how density-driven adipocyte commitment influences gene expression pattern in ASCs, primary ASCs cultured under sub-confluent and confluent conditions for 2 (2d) and 12 days (12d) were harvested (Fig. 1A). Total RNA samples were subjected to microarray analyses using Affymetrix HG-U133A GeneChips™. The cyber-t test was applied to identify those genes whose expression was significantly different between the sub-confluent and confluent cultures at each time point. Using a comparison criteria matrix, we identified a total of 1612 and 1289 genes that were differentially expressed in 2d and 12d confluent cultures in comparison to their sub-confluent counterpart, respectively (Supp. Table 1). Approximately half of the confluency-affected genes were down-regulated and the other half up-regulated (Fig. 1B). A careful examination of the heat map revealed several interesting patterns among the differentially expressed genes. The majority of the down-regulated genes appeared to be fully repressed at the early time point of confluency (2d). In contrast, a large number of up-regulated genes displayed gradual increases in mRNA levels, peaking at the 12d time point (group a in Fig. 1B). The up-regulated genes in groups b and c were maximally induced at the 2d time point, and their mRNA levels either remained high (group b) or regressed at the 12d time point (group c). The potential relationship between the “early” versus “late” response genes was addressed in our later study (see below).

Gene Ontology (GO) analysis was performed to identify potential cellular structure and functions that were affected by cell density. As expected for confluent primary cell culture, the largest group of down-regulated genes at 2d consists of cell cycle regulatory genes (Fig. 1C). In addition, genes that are involved in intracellular organelle and cytoskeleton functions are also highly enriched in the down-regulated gene group. On the other hand, many of the up-regulated genes at the 2d point are related to signal transduction, ECM, and cell adhesion (Fig. 1D). For example, genes involved in prostaglandin metabolism (PTGFR and PTGDS2), cell growth (EGF and IGF-1), inflammation (SOD-2 and IL-8), and Wnt signaling (WISP-2) were substantially induced. There is a large overlap of functional groups between the 2d and 12d time points (compare Fig. 1C with 1E, and 1D with 1F). However, proteinase-encoding genes were specifically enriched in the 2d up-regulated gene group, whereas panels of fibrinogen and wound response genes were enriched only at the 12d time point for the up-regulated gene group. Together, these data suggest that the confluent condition exerts a global effect on the expression of genes with significant signaling functions in ASCs.

Validation of microarray data by RT-PCR

Next, we performed quantitative RT-PCR analysis to validate the microarray data, with the focus on a number of genes that were previously implicated in ASC functions (Fig. 2). Among the down-regulated genes, ID-3 is a dominant negative member of the helix-loop-helix (HLH) family (Fig. 2A); and Nurr1 is an orphan nuclear receptor (Fig. 2B). Both genes have been implicated in negative regulation of adipogenesis [43,51]. Breast tumor suppressor BRCA1 is another down-regulated gene verified by our RT-PCR study. We and others recently showed that BRCA1 is an important negative regulator of aromatase expression in ASCs and ovarian granulosa cells [48,52,53]. During the high-density treatment, a marked decrease of BRCA1 mRNA (Fig. 2C) was accompanied with a significant induction of aromatase (Fig. 2D). The effects of confluency on several up-regulated genes were also confirmed by RT-PCR. These include genes that encode growth factors IGF-1 (Fig. 2E) and FGF-7 (Fig. 2F), the Wnt signaling protein WISP2 (Fig. 2G), and suprabasin (SBSN), a cell adhesion factor that is involved in epidermal differentiation [54].

Individual-based variation in confluency-induced gene expression

To determine whether primary ASCs from different individuals share similar confluency-triggered gene expression profiles, we collected a cohort of ASCs from a number of healthy individuals who underwent abdominal liposuction. When cultured in adipogenesis-inducing medium, confluent ASCs from a cohort of 11 different donors differentiated into mature adipocytes with similar efficiency [55] (also see Supp. Fig. 1A). However, the cell density-induced mRNA levels of aromatase and IGF-1 differed substantially among the different ASC samples (Supp. Fig. 1B and 1C). To conduct a more thorough investigation, we chose two ASC samples that represent the high and low end in the spectrum of aromatase inducibility. h6-07L was the ASC sample used in the current microarray study that exhibited significant aromatase gene induction, whereas confluent h6-06L had very low levels of aromatase. Upon adipogenic induction, both samples gave rise to similar percentages of Oil-Red-O stained adipocytes (Fig. 3A). Adipogenic markers PPAR γ and C/EBP α were also induced in both ASC samples, with somewhat higher expression in h6-07L cells (Fig. 3B). When cultured under the high-density condition, the mRNA levels of BRCA1 and ID-3 in both ASC samples were rapidly repressed with similar kinetics (Fig. 3C and 3D). In contrast, there was a huge difference in the magnitude of confluency-induced gene expression from these two ASC samples. Unlike confluent h6-07L, h6-06L did not express any significant amount of IGF-1 (Fig. 3E), aromatase (Fig. 3F), or SBSN (Fig. 3G) in response to high cell density treatment. Therefore, the adipogenic capability of ASCs is not necessarily proportional to the confluency-triggered inducibility of endocrine-related genes.

Foxo1 is a key player during confluency-induced gene activation

In order to identify the probable transcription factors that confer confluency-induced gene activation, we used an online motif-finding tool, Whole Genome rVisa [47], to conduct a computational search for binding sites of common transcription factor among the promoter regions of these genes. To achieve high specificity, we searched for binding sites that are not only significantly enriched in the 1kb promoter region of the up-regulated genes as compared to that of all the genes in the human genome, but also those that are conserved between human and mouse (see Materials and Methods). Approximately 30% of the top 300 up-regulated genes that were transcriptionally activated at the 2d time point (early response) were predicted to possess Foxo1/Foxo4 binding sites (p -value $< 5E-8$) (Supp. Table 2). Similar results were obtained with fewer or all early response genes. Also, the enrichment of the Foxo1/Foxo4 binding sites became more significant when we searched the 500bp rather than 1kb promoter regions (p -value $< 1E-12$), suggesting that Foxo1/Foxo4 may preferentially bind to proximal promoter regions. Interestingly, the Foxo1/Foxo4 binding sites are not significantly enriched among the late response genes ($p > 0.01$), which are defined as those genes that are up-regulated at the 12d, but not 2d, time point.

To determine whether the Foxo proteins are functionally involved in activation of early response genes, we knocked down Foxo1 by siRNA (Fig. 4A and 4B) and assessed the effect on the expression of a number of early response genes that contain the Foxo1 binding motifs. Depletion of Foxo1 dramatically reduced the basal as well as induced levels of SBSN (Fig. 4C), SFRP4 (Fig. 4D), FGF-7 (Fig. 4E), and SPON1 (Fig. 4F). Interestingly, Foxo1 depletion only abrogated the confluency-induced, but not basal mRNA level of IGF-1 (Fig. 4G). Angiopoietin-like 1 (ANGPTL1) was the only Foxo motif-containing gene examined whose expression was not affected at all by Foxo1 knockdown (Fig. 4H). Taken together, our data strongly suggest that Foxo1 serves as a master activator of most confluency-induced genes in ASCs.

SBSN is required for density-dependent stimulation of aromatase expression and tumor cell migration

In light of the distinct waves of transcriptional response to cell confluency, we hypothesized that expression of some of the early response factors might in turn mediate stimulation of the late response genes. Aromatase, one of the late response genes (Fig. 2D and 3F), plays a critical role in stromal estrogen biosynthesis and serves as an important target in endocrine therapy for ER-positive postmenopausal breast cancer. To test whether the confluency-induced aromatase expression was affected by some of the early response genes, we individually knocked down three early response genes that are either known or predicted to encode secretory factors (FBLN1 and SBSN) [54,56] or membrane-associated factor (STON2) [57]. As shown in Fig. 5A, the corresponding siRNA oligos can efficiently knock down all three early response genes. However, only depletion of SBSN significantly reduced the aromatase mRNA under the confluent condition (Fig. 5B). Given that SBSN was positively regulated by Foxo1 (Fig. 4C), we predicted that Foxo1 would promote aromatase transcription through its action on SBSN. Indeed, Foxo1 knockdown resulted in a substantial reduction of the aromatase mRNA level in confluent ASCs, without affecting the basal level of aromatase mRNA in sub-confluent cells (Fig. 5C). Thus, Foxo1 and SBSN most likely act upstream in the confluency-triggered signal transduction pathway to activate aromatase expression and local estrogen production in adipose tissue.

In addition to its importance in local estrogen production and development of estrogen-dependent breast cancer, ASCs can also promote estrogen-independent tumor growth via various ASC-secreted factors such as cytokines [33,35]. For example, we showed recently that conditioned medium from confluent ASCs substantially stimulated the migratory capability of ER-negative breast cancer cells MDA-MB-231 [33]. To determine whether Foxo1 or SBSN is involved in ASC-stimulated tumor cell migration, confluent ASCs with control, Foxo1, and SBSN knockdown were compared for their ability to promote migration of MDA-MB-231 in a Boyden chamber assay. Consistent with our recently published work [33], ASCs that were transfected with two control siRNA oligos (siControl and siACTC1) exerted significant stimulatory effects on tumor cell migration (Fig. 5D). In contrast, Foxo1 or SBSN knockdown significantly reduced the capability of ASCs to promote tumor cell migration. As MDA-MB-231 cells are estrogen-independent, we infer that the Foxo1 and SBSN-dependent effect on tumor cell migration is distinct from their roles in activation of aromatase gene expression.

DISCUSSION

It is well documented that adipose tissue has enormous impetus in normal physiology and disease progression via its capability of secreting a number of growth and inflammatory factors [58]. Therefore, it is important to identify the underlying regulatory mechanisms that can induce the production of key factors from different components of adipose tissue. Our study revealed that ASCs under a high-density condition underwent significant changes in transcriptional regulation, which results in marked increases in the expression of a number of endocrine-related factors. In addition, our findings suggest an important function of Foxo1 in directly or indirectly controlling the early and late response gene expression during the density-driven process. These findings lead us to a model whereby adipocyte commitment can significantly augment the endocrine and paracrine output of adipose tissue through activation of consecutive waves of transcriptional programs, which may ultimately enhance the tumor-promoting ability of adipose tissue.

Various members of the Foxo family are expressed and functionally important in adipose tissue [59,60]. In particular, expression and activation of Foxo1 is temporally regulated during adipogenesis. Constitutively active Foxo1 blocks differentiation of preadipocytes, whereas a dominant-negative mutant of Foxo1 promotes adipogenesis [61], suggesting a negative role of

the transcriptional regulator in adipogenesis. Furthermore, Foxo1 appears to play an important role in linking insulin-mediated signal transduction and adipogenesis-related transcriptional activation in adipose tissue [61,62]. Our microarray and gene-specific study extends the previous findings by demonstrating the requirement of Foxo1 for initiating a confluency-dependent transcriptional cascade that leads to elevated production of many inflammatory and endocrine-related factors.

The mechanism of density-triggered activation of Foxo1 remains to be elucidated. There was only a moderate increase in Foxo1 mRNA following the high-density treatment (Fig 4A). Acetylation or phosphorylation of Foxo1 is known to regulate its transcriptional activity [63]. In addition, Foxo1 may regulate the transcription of its target promoters by partnering with other site-specific transcription factors, the levels of which may increase in response to high cell density. Of note, C/EBP β is a transcription activator that can physically and functionally interact with Foxo1 [64,65]. Furthermore, our previous study indicates that density-induced protein level of C/EBP β leads to activation of aromatase transcription in ASCs [34].

Tissue- and cell type-restricted aromatase transcription is driven by a number of tissue-specific transcription promoters that are located in a 90-kilobase genomic region upstream of the aromatase-coding sequence [34,66]. We have recently shown that cell density-induced aromatase transcription in ASCs is through the activation of two breast cancer-associated promoters (I.3 and PII) [34]. The current study indicates that confluency-induced activation of SBSN by Foxo1 is required for subsequent stimulation of aromatase transcription. While Foxo1 knockdown significantly reduces confluency-induced aromatase transcription in ASCs, no apparent Foxo1 binding sites can be found in the upstream sequences proximal to the aromatase encoding region. Furthermore, unlike the Foxo1-regulated early response genes, the density-triggered increase in the aromatase mRNA level was only prominently induced days after ASCs were seeded under the confluent condition. Although a possible direct impact of Foxo1 on aromatase transcription cannot be excluded at this point, we favor the alternative scenario whereby Foxo1 leads to the activation of aromatase transcription through its activation of the SBSN gene.

SBSN is a secreted protein that plays a role in adhesive function and epidermal differentiation [67]. The novel function of SBSN in confluency-associated aromatase transcription raises the distinct possibility that SBSN may be involved in the regulation of local estrogen biosynthesis and subsequently breast cancer development. As both skin and adipose tissue serve as important extragonadal sites for aromatase expression and estrogen biosynthesis, it will be of importance to determine whether SBSN is a common regulatory factor for aromatase transcription in both tissues. It is also tempting to speculate that neutralization of SBSN activity in these peripheral tissues may help dampen local estrogen biosynthesis and thus reduce breast cancer risk among postmenopausal women.

Overall expansion of adipose tissue mass is associated with increases in adipocytes as well as their precursor cells including ASCs [68]. It is conceivable that increase in cell number in adipose tissue leads to enhanced cell-cell contact and significant cell shape changes, a situation probably akin to confluent ASCs cultured *in vitro*. It will be important to determine whether the gene expression profile as observed in confluent ASC culture recapitulates that in adipose tissue of obese individuals. As additional stromal cell types and ECM likely contribute to stromal gene regulation in adipose tissue, it will also be of interest to incorporate these integral components of adipose tissue in future investigation.

Despite comparable adipogenic capability, ASC samples isolated from different individuals display significant difference in the inducibility of various secreted factors, some of which have been implicated in the development of metabolic dysfunction and cancer (e.g. IGF-1 and

aromatase). This suggests that at least part of the genetic determinants for confluency-induced gene expression is distinct from those for adipocyte commitment. The individual-based variation in the inducibility of these endocrine-related genes may contribute to disease susceptibility. Identification of the molecular basis for the inducibility of ASC-secreted factors may lead to the development of novel risk-assessing tools and therapeutic targets for adipose tissue-associated diseases.

CONCLUSIONS

The present study describes the potential of multipotent ASCs in producing cancer-related endocrine factors under the confluent tissue culture condition. Our data suggest that high cell density triggers a cascade of gene activation events, with some of the “early response” genes in turn stimulating “late response” ones. Our bioinformatics and functional interrogation further implicate Forkhead transcription factors in initiating the confluency-triggered transcriptional cascade that led to elevated production of many inflammatory and endocrine-related factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

TNF- α	Tumor necrosis factor- α
IL-6	Interleukin-6
MCP-1	Monocyte chemotactic protein-1

ASC	Adipose derived stem cell
CCL5	Chemokine (C-C motif) ligand 5
PPAR γ	Peroxisome proliferator-activated receptor γ
C/EBP α	CAAT/enhancer binding protein α
C/EBP β	CAAT/enhancer binding protein β
WISP2	WNT1 inducible signaling pathway protein 2
Foxo1	Forkhead box O1
Foxo4	Forkhead box O1
SBSN	Suprabasin
ACTC1	actin, alpha, cardiac muscle 1
PTGFR	Prostaglandin F receptor
PTGDS2	Prostaglandin D2 synthase 2
SOD2	Superoxide dismutase 2
IL-8	Interleukin-8
SFRP4	Secreted frizzled-related protein 4
FGF-7	Fibroblast growth factor-7
SPON1	Spondin1
FBLN1	Fibulin1
Angptl1	Angiopoietin-like 1
ID-3	Inhibitor of DNA binding 3
STON2	Stonin-2

Figure 1.

Microarray analysis of confluency-triggered gene expression in ASCs. **(A)** Representative images of ASCs that grew under sub-confluent condition for 2 days (Sub-conf) and under confluent condition for 2 (2d conf) and 12 days (12d conf). **(B)** Hierarchical clustergram for confluency-regulated gene expression in ASCs. Low and high mRNA levels are indicated by blue and red colors, respectively. Intermediate expression levels are designated as white. Sub-groups a-c among the up-regulated genes display different kinetics of transcriptional activation. **(C-F)** Gene ontology analysis reveals several biological pathways that are enriched with confluency-triggered up- and down-regulated genes at the 2d (C and D) and 12d time points (E and F).

Figure 2.

Verification of microarray results by RT-PCR. Relative mRNA levels of various genes as revealed by the microarray analysis were measured by quantitative RT-PCR, using cDNA samples from sub-confluent and confluent ASC samples of different time points (2d and 12d). These include down-regulated genes Id3 (A), Nurr1 (B), and BRCA1 (C); and up-regulated genes aromatase (D), IGF-1 (E), FGF-7 (F), WISP-2 (G), and SBSN (H). Data represent mean of 3 individual experiments; and error bars (standard deviation) are included in each graph (*, $P < 0.01$ versus sub-confluent).

Figure 3.

Individual-based variation in confluency-induced gene expression. (A) ASCs isolated from two individuals (h6-07L and h6-06L) exhibited similar degrees of adipogenesis as measured by Oil-Red-O staining. (B) Induction of PPAR γ and C/EBP α in the two ASC samples upon adipogenesis. (C-G) Comparison of mRNA levels of several confluency-affected genes in the two ASC samples that were cultured under sub-confluent and confluent conditions at different time points (2d and 12d). Genes included are BRCA1 (C), Id3 (D), IGF-1 (E), aromatase (F), and SBSN (G). Data represent mean of 3 individual experiments; and error bars (standard deviation) are included in each graph (*, $P < 0.01$ versus sub-confluent).

Figure 4.

Foxo1 is required for transcriptional activation of multiple confluency-induced early response genes in ASCs. **(A-B)** Foxo1 was efficiently knocked down in ASCs by Foxo1-specific siRNA oligos, as indicated by qRT-PCR (A) and Western analysis (B). A non-targeting siRNA (siControl) was used as a control for the knockdown experiment. α -Tubulin was used as a loading control for the Western blot analysis. **(C-H)** Relative mRNA levels of multiple early response genes were assessed by RT-PCR in the siControl and siFoxo1-treated cells under both sub-confluent and 2d confluent culture conditions. These genes include SBSN (C), SFRP4 (D), FGF7 (E), SPON1 (F), IGF-1 (G), and ANGPTL1 (H). Data represent mean of 3 individual experiments; and error bars (standard deviation) are included in each graph (*, $P < 0.01$ versus control).

Figure 5.

Foxo1 controls aromatase transcription via transcriptional regulation of SBSN. (A) FBLN1, STON2, and SBSN were individually knocked down in ASCs. Their relative mRNA levels in the knockdown versus the control cells were measured by RT-PCR. (B) The effects of siRNA knockdown of various genes on aromatase gene expression were measured by RT-PCR in sub-confluent and confluent ASCs (4 day). (C) The effect of Foxo1 knockdown on aromatase mRNA levels was determined using siControl and siFoxo1-treated ASCs that were cultured under sub-confluent and confluent conditions. (D) Foxo1 or SBSN knockdown reduces the ability of ASCs to stimulate migration of breast tumor cells. siControl and siACTC1 are two control siRNA oligos used in the experiment. Data represent mean of 3 individual experiments; and error bars (standard deviation) are included in each graph.