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Genes that integrate multiple adipogenic signaling pathways in human mesenchymal stem cells

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ABSTRACT

Adipogenesis is a well-characterized cell differentiation process. A large body of evidence has revealed the core transcription factors and signaling pathways that govern adipogenesis, but cross-talks between these cellular signals and its functional consequences have not been thoroughly investigated. We, therefore, sought to identify genes that are regulated by multiple signaling pathways during adipogenesis of human mesenchymal stem cells. Focusing on the early stage of adipogenesis, microarray analysis and quantitative RT-PCR identified 12 genes whose transcription levels were dramatically affected by the complete adipogenic induction cocktail but not by the cocktail's individual components. Expression kinetics of these genes indicate diverse mechanisms of transcriptional regulation during adipogenesis. Functional relationships between these genes and adipogenic differentiation were frequently unknown. This study thus provided novel adipogenic gene candidates that likely mediate communications among multiple signaling pathways within human mesenchymal stem cells.

1. Introduction

In vitro adipogenesis is one of the most attractive cell systems to study global behavior of intracellular signaling networks that has pharmaceutical relevance [1–3]. A simple *in vitro* protocol drives adipogenesis of various pre-adipocyte cell lines and mesenchymal stem cells in an efficient and reproducible manner. Using this standard protocol, pre-adipocyte cell lines, such as 3T3-L1, differentiate when dexamethasone (Dex), insulin, and 3-isobutyl-1-methylxanthine (IBMX) are added to the media [4–6]. Human mesenchymal stem cells (hMSCs), on the contrary, require these three reagents plus indomethacine (Indo) for adipogenesis [7]. Cells treated with these adipogenic cocktails develop cellular features that characterize white adipocytes *in vivo*, such as lipogenesis, insulin-stimulated glucose uptake, and adipokine secretion. A detailed understanding of the signaling networks that drive *in vitro* adipogenesis, therefore, will likely offer strategies and drug targets for management of obesity and other metabolic syndromes [8–10].

The core transcription factors and signaling pathways that drive adipogenesis have been established [1,11,12]. Successive expression of CCAAT/enhancer-binding protein (C/EBP) family members triggers expression of peroxisome proliferator-activated receptor gamma ($PPAR_\gamma$), the master adipogenic transcription factor. $PPAR_\gamma$ is essential for adipogenesis both *in vitro* and *in vivo* [1,13]. The $PPAR_\gamma$-C/EBP pathway is regulated by major intracellular signaling pathways that include Wnt [14–16], phosphoinositide 3-kinase (PI3K)/Akt [17–19], mitogen-activated protein kinase (MAPK) [20,21], transforming growth factor-β (TGF-β)/Smad [22,23], and Hedgehog [24]. These signaling pathways often control adipogenesis cooperatively, although the challenge has just began to understand how multiple signals are integrated in this context, and exactly what functional consequences result from signal integration [22].

To characterize signaling pathway cross-talk during adipogenesis, we sought to identify genes that are regulated by multiple

Abbreviations: hMSCs, human mesenchymal stem cells; Dex, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; C/EBP, CCAAT/enhancer-binding protein; $PPAR_\gamma$, peroxisome proliferator-activated receptor gamma; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; TGF, Transforming growth factor; AOC3, amine oxidase, copper containing 3; CA2, carbonic anhydrase II; HSD11B1, hydroxysteroid (11-beta) dehydrogenase 1; RGS2, regulator of G-protein signaling 2; STAR, steroidogenic acute regulator, nuclear gene encoding mitochondrial protein; SLCO2A1, solute carrier organic anion transporter family, member 2A1; KCNK3, potassium channel, subfamily K, member 3; MT1JP, metallothionein 1J (pseudogene); C14orf162, chromosome 14 open reading frame 162; NOX4, NADPH oxidase 4.

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pathways. Inspired by Liu et al. [25] and Pantoja et al. [26], we performed microarray analysis on hMSCs treated either with the full adipogenic cocktail, or single cocktail components. Genes of interest were transcriptionally affected only when the full adipogenic cocktail was applied. Each cocktail component seems to activate distinct signaling pathways in hMSCs, since efficient adipogenesis indeed results from synergetic interactions among these stimuli [25–28]. We reasoned, therefore, that genes whose expression levels are affected only with the complete cocktail represent interfaces between multiple pathways in the adipogenesis-related signaling network, and likely play key roles in fat-cell differentiation. Following microarray analysis, candidate genes were confirmed by quantitative RT-PCR. Finally, to understand the kinetics of signaling pathway interactions in this context, transcription levels of affected genes were measured during an adipogenic time course.

2. Materials and methods

2.1. Cell culture and differentiation

hMSCs (CD105-, CD166-, CD29- and CD44-positive; <5% positive for CD14, CD34 and CD45) were purchased from Lonza (PT-2501, East Rutherford, NJ, USA). hMSCs were maintained in MSC basal medium (MSCGM BulletKit, Lonza). hMSCs were expanded to passage 4 before experiments. Sub-confluent or confluent cultures of cells were treated and maintained in adipogenic induction medium (hMSC Differentiation BulletKit, Adipogenic, Lonza) until RNA extraction or microscopic evaluation. Cells were stimulated with Dex, insulin, Indo, or IBMX alone using the same concentration as found in the adipogenic induction medium.

2.2. Total RNA extraction

hMSCs were maintained in MSC basal medium (the control), or MSC basal medium plus Dex, or insulin, or IBMX, or Indo, or all components together (the adipogenic cocktail) for 24 h before total RNA extraction. Total RNA was extracted from hMSCs using the RNeasy kit (Qiagen), and treated with RNase-free DNase I (Qiagen) during extraction.

2.3. Microarray screening

Each RNA sample except for insulin and Indo samples was labeled with Cy3, and hybridized to an Agilent Whole Human Genome Microarray 4x44K (G4112F) chip according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent Technologies), and signal intensities calculated using GeneSpring GX software (Agilent Technologies). The experiment was performed in duplicate. Expression fold changes were calculated using Excel.

2.4. Quantitative RT-PCR

cDNA was prepared using the PrimeScript kit (Takara). In each case, random and oligo(dT) primers were used. Quantitative PCR analysis was performed with a Thermal Cycler Dice Real-Time System (Takara), using SYBR Premix EX Taq (Takara) as the reaction reagent. Results were analyzed based on the second derivative method, and cycle threshold (Ct) values were normalized to 18S ribosomal RNA. Primer sequences are listed in Table S1.

3. Results

Confluent hMSCs were placed into media supplemented with: (1) the complete adipogenic cocktail (Dex, Insulin, Indo, and IBMX) (ALL); (2) Dex alone; (3) insulin alone; (4) Indo alone; (5) IBMX alone; or (6) no adipogenic components (NONE). On day 13, light microscopy was used to assess lipid cell formation in these samples. A high percentage of cells that accumulated visible lipid-containing vesicles insides were detected only with the complete cocktail, whereas Dex alone resulted in a small number of lipid cells. No lipid vesicle formation were observed with any other media (data not shown). These results confirm that efficient hMSC adipogenesis occurs only with the full cocktail (all four components).

To evaluate gene expression levels, total RNA was extracted from these six hMSC samples at 24 h after the reagent treatment started. The reason for this RNA sampling time point was because signaling pathways crucial to adipogenesis likely began activated at this time, which was shown by elevating expression of the master adipogenic transcription factor, $PPAR_{\gamma}$, in the ALL sample (Fig. S1A). As we have previously shown, Dex alone also induced a slight increase in $PPAR_{\gamma}$ expression, but dramatic up-regulation of $PPAR_{\gamma}$ at 24 h only occurred with the complete adipogenic cocktail (Fig. S1B) [29]. We are confident, therefore, that by focusing on the 24 h time point we will effectively identify genes that respond to major adipogenic signaling pathways in hMSCs.

Four hMSC treatment conditions (ALL, Dex, IBMX or NONE) were subjected to microarray analysis, using the Agilent Whole Human Genome Microarray 4x44K. The entire process, from RNA extraction to microarray screening, was performed in duplicate. A total of 150 and 158 probes (corresponding to 102 and 83 genes, respectively) demonstrated greater than 10-fold up- or down-regulation, respectively, when the ALL and NONE samples were compared. Genes were included only if results from the two arrays were in agreement. Interestingly, out of the obtained genes, many more genes than we expected were also affected when only Dex or IBMX was added to the medium (gray circles in Fig. 1A–D). Thus, genes were only collected if a 10-fold greater expression change was measured for ALL than for any single component sample. For example, of the 150 probes up-regulated by ALL, a small fraction of these genes were 10-fold less affected by Dex (37 and 41 probes (corresponding to 29 and 26 genes, respectively) on the 1st and 2nd arrays, respectively) (Fig. 1A and B), and 24 of these genes were finally identified on both arrays. This result demonstrates that by considering transcriptional responses to single cocktail components, our method identifies genes whose expression levels strongly correlate with adipogenic differentiation.

The same data analysis strategy revealed 67 genes that were up-regulated by ALL 10-fold more than by IBMX, and genes that were down-regulated by ALL 10-fold more than by Dex or IBMX alone (21 and 17 genes, respectively). Taken together, this microarray analysis identified 17 genes that were regulated by ALL, but significantly less affected by either Dex or IBMX alone (16 genes up-regulated, and 1 gene down-regulated) (Fig. 1E and F). Finally, these 17 genes were subjected to quantitative RT-PCR analysis using all 6 RNA samples. This test confirmed that 12/17 genes changed transcriptional levels by more than 10-fold at 24 h with ALL compared with any one component or NONE (Fig. 2 and Table 1).

Some of the genes identified by this method have well-defined roles in adipogenesis, or adipocyte biology. These genes include hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1), regulator of G-protein signaling 2 (RGS2), and amine oxidase, copper AAAAAAcontaining 3 (AOC3). HSD11B1 controls glucocorticoid levels, and is regulated by C/EBP proteins in murine adipose cells [30]. HSD11B1 knockout mice have increased levels of $PPAR_{\gamma}$ and adiponectin in adipose tissue [31]. RGS2 cooperatively promotes adipogenesis with a specific ligand for $PPAR_{\gamma}$ [32]. Finally, AOC3 is the major plasma membrane protein of murine and human adipocytes [33]. Furthermore, 3 additional genes identified in this