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MECHANISMS OF DEVELOPMENT

Downregulated expression of the secreted glycoprotein follistatin-like 1 (Fstl1) is a robust hallmark of preadipocyte to adipocyte conversion

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ABSTRACT

Obesity is a public health crisis in the United States. Targeting preadipocyte to adipocyte conversion may be an effective approach to regulate adipose mass. Using differential screening we identified Fstl1, a secreted glycoprotein with roles in immunomodulation, cell growth, cardioprotection, and vascularization, as a "preadipokine". Fstl1 is highly expressed in 3T3-L1 preadipocytes and dramatically downregulated early in their differentiation to adipocytes. Northern blot analysis of murine tissues reveals white adipose tissue (WAT), lung and heart as primary sites of Fstl1 transcript expression. In WAT, Fstl1 transcript is restricted to the preadipocyte-containing stromal-vascular cell population. Time course studies in multiple adipogenesis models reveal downregulation of Fstl1 is a hallmark of white and brown adipocyte conversion. By Western blot, we show culture media of 3T3-L1 preadipocytes contains high levels of Fstl1 protein that rapidly decline in adipocyte conversion. Moreover, we observe a correlation between preadipocyte phenotype and Fstl1 expression in that TNFα-mediated de-differentiation of 3T3-L1 adipocytes is accompanied by re-expression of Fstl1 transcript and protein. Treatment of 3T3-L1 preadipocytes with a panel of 18 hormones and other agents revealed the demethylating agent 5-aza-cytidine decreases Fstl1 transcript and protein levels by ~90%. Furthermore, of 10 additional preadipocyte-expressed genes analyzed we find Pref-1, Col1A1, Sca-1/Ly6a, Lox and Thbs2, are also downregulated by 5-aza-cytidine. Using luciferase reporter constructs containing 791 or 3922 bp of the Fstl1 5′ flanking region, we determine negative transcriptional regulation by Kruppel-like factor 15. Together, our data suggest downregulation of Fstl1 expression may be an important feature of preadipocyte to adipocyte conversion.

1. Introduction

Obesity can lead to multiple of health problems including non-insulin dependent diabetes mellitus, hypertension, cardiac infarction and some types of cancers (Flier, 2004; Ntambi and Young-Cheul, 2000; Spiegelman and Flier, 2001). Dysregulation of multiple aspects of adipose tissue and adipocyte function is a hallmark of obesity and its co-morbidities (Flier, 2004; Greenberg and Obin, 2006; Lazar, 2005; Qatanani and Lazar, 2007; Spiegelman and Flier, 2001). White adipose tissue (WAT) is a unique organ that regulates the balance between energy storage and release to maintain energy homeostasis in accord with nutritional status (Zechner et al., 2009). WAT consists of a combination of adipocytes, small blood vessels,

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nerve tissue, fibroblasts, preadipocytes, stem cells, and other undefined cell types (Geloen et al., 1989; Zuk et al., 2002). WAT is now recognized as a dynamic secretory endocrine organ (Ailhaud, 2006; Breitling, 2009; Waki and Tontonoz, 2007). A number of soluble factors and cytokines including leptin, adiponectin, resistin and TNFα are synthesized and secreted by white adipocytes (Maeda et al., 2002; Spiegelman and Flier, 1996; Steppan et al., 2001). Recent studies in humans indicate that for a given individual the number of adipocytes within WAT is largely constant during adulthood (Spalding et al., 2008). However these studies also revealed that adipocytes within WAT turnover at a rate of ~10% *per* year (Spalding et al., 2008) and are presumably maintained at constant numbers *via* ongoing differentiation of preadipocytes to adipocytes.

Adipogenesis is the process of the formation of new adipocytes from preadipocyte precursors, and is thus important for the understanding and control of WAT function and obesity (Lefterova and Lazar, 2009). To date, this process has been studied primarily using *in vitro* cell culture models. 3T3-L1 preadipocytes, developed several decades ago by Green and colleagues, are the best characterized model of *in vitro* adipogenesis and are extensively utilized for studies of the molecular mechanisms of adipogenesis (Green and Kehinde, 1974, 1975; Green and Meuth, 1974). 3T3-L1 preadipocytes were cloned from Swiss 3T3 cells, a cell type originally derived from disaggregated late-stage mouse embryo (Green and Kehinde, 1974). While 3T3-L1 preadipocytes exhibit a low degree of spontaneous adipogenesis, their standard *in vitro* differentiation involves a 2-day treatment of post-confluent cells with the adipogenic agents dexamethasone (Dex) and methylisobutylxanthine (Mix), which greatly enhances extent and uniformity of differentiation (Rubin et al., 1978). The adipogenic transcription factors C/EBPs (CCAAT/enhancer binding proteins) and PPARγ (peroxisome proliferator-activated receptor-gamma) play key roles in the transcriptional cascade governing adipogenesis (Gregoire, 2001; Gregoire et al., 1998; Lazar, 2005; Lefterova and Lazar, 2009; Rajala and Scherer, 2003; Rosen et al., 2002; Rosen and Spiegelman, 2001). The nature of PPARγ as a master regulator in adipogenesis has been documented in numerous studies, including the observation that forced expression of PPARγ, in the presence of a PPARγ ligand, stimulates adipogenesis in fibroblast cell lines (Hu et al., 1995; Lazar, 2005; Shao and Lazar, 1997; Tontonoz et al., 1994). C/EBPβ and C/EBPδ transcription factors are rapidly induced following exposure to adipogenic agents to impact expression of PPARγ and C/EBPα (Rosen and Spiegelman, 2001; Tontonoz et al., 1994) and C/EBPα cooperates with PPARγ to induce a maximal adipogenic response (Hu et al., 1995). The interplay of an expanding and complex network of various types of regulatory signals ultimately leads to the gene expression profile and phenotype of mature lipid-laden terminally differentiated adipocyte. In many cases, expression of genes that define the mature white adipocyte are not readily detected at the preadipocyte stage, for example those functioning in aspects of insulin signaling, lipid metabolism, lipid droplet function, and numerous adipokines (Gregoire, 2001; Gregoire et al., 1998; Rajala and Scherer, 2003).

A number of large-scale microarray profiling studies of transcriptional changes during 3T3-L1 *in vitro* adipogenesis have documented upwards of hundreds of genes whose expression is dramatically increased or decreased (Burton et al., 2002, 2004; Burton and McGehee, 2004; Guo and Liao, 2000; Ross et al., 2000, 2002; Soukas et al., 2000, 2001). Many studies have characterized expression and function of genes whose expression is absent/low in preadipocytes and dramatically upregulated in adipocyte conversion (Gregoire, 2001; Gregoire et al., 1998; Lefterova and Lazar, 2009; Rajala and Scherer, 2003; Rosen and Spiegelman, 2001). In marked contrast, only a few genes with highly selective expression in preadipocytes vs. mature adipocytes have been studied in any detail (Harp, 2004). These include the transmembrane protein Pref-1 (preadipocyte factor 1), which is active as a processed soluble factor (Smas and Sul, 1993), the secreted factor Wnt10b (Ross et al., 2000), GATA-2 and GATA-3 transcription factors (Tong et al., 2000). Each of these acts as important preadipocyte-expressed inhibitory regulators of adipogenesis, as demonstrated by *in vivo* and *in vitro* studies. Pref-1 transcript is highly expressed in 3T3-L1 preadipocytes and absent in adipocytes (Smas and Sul, 1993) and is also present, albeit at markedly lower levels (Soukas et al., 2001), in the preadipocyte-containing stromal-vascular cell population *in vivo* (Zhou et al., 1999). Constitutive expression or addition of soluble Pref-1 inhibits 3T3-L1 adipocyte conversion (Smas et al., 1997; Smas and Sul, 1993). Pref-1-null mice exhibit obesity (Moon et al., 2002) and Pref-1 null mouse embryo fibroblasts exhibit enhanced differentiation to adipocytes (Kim et al., 2007b; Moon et al., 2002). In the canonical Wnt signaling pathway, Wnts bind to transmembrane Frizzled family receptors leading to stabilization of soluble β-catenin. β-Catenin translocates to the nucleus to complex with TCF/LEF transcription factor leading to expression of genes inhibitory for adipogenesis (Prestwich and Macdougald, 2007). Overexpression of Wnts, for example Wnt10b, or stabilizing free cytosolic β-catenin blocks adipogenesis (Ross et al., 2000). GATA-2 and GATA-3 transcription factors are downregulated during adipocyte differentiation and their constitutive expression traps cells at the preadipocyte stage (Tong et al., 2000). A recent study suggests that certain preadipocyte-expressed genes may not only act to block adipogenesis when constitutively expressed in preadipocytes but, under some circumstances, can modulate the phenotype of mature adipocytes. In this case, knockdown of PPARγ in concert with ectopic expression of GATA-3 in 3T3-L1 adipocytes resulted in mature adipocytes taking on aspects of a dedifferentiated transcriptional profile (Schupp et al., 2009). In addition to the long-standing use of *in vitro* adipogenesis models, new investigations have made significant contributions towards the study of preadipocyte populations *in vivo* (Park et al., 2008). Until recently, such studies were severely limited due to lack of molecular markers and other tools for their identification and study. Cell lineage tracing approaches developed by Graff and coworkers demonstrated that white preadipocyte progenitor cells reside in the vasculature of adipose tissue (Tang et al., 2008). Friedman and colleagues reported that a subpopulation of early adipocyte progenitor cells, obtained from murine WAT and demonstrating Lin(−):CD29(+):CD34(+):Sca-1(+):CD24(+) surface marker expression, are capable of *de novo* formation of WAT upon injection into residual fat pads of lipodystrophic mice (Rodeheffer et al., 2008). However the process of emergence

of functionally distinct preadipocytes from mesenchymal and/or other stem cell populations, in both the *in vitro* and *in vivo* setting, remains obscure. A further elucidation of the molecular definition of preadipocytes is essential to a full understanding of adipogenesis. Additionally, the identification of new genes preferentially downregulated upon preadipocyte to adipocyte conversion may uncover novel and important regulatory roles for preadipocyte-enriched genes.

The follistatin-like-1 (Fstl1) gene, alternately named FRP (Zwijsen et al., 1994), Flik (Patel et al., 1996), Occ1 (Tochitani et al., 2001) and TSC-36 (Shibanuma et al., 1993), encodes a secreted glycoprotein of ~37 kDa. The Fstl1 cDNA was first isolated in a differential screening for TGF-β1-inducible genes in the MC3T3-E1 murine osteoblastic cell line (Shibanuma et al., 1993). The presence of an extracellular calcium-binding type region places Fstl1 in the BM-40/SPARC/osteonectin protein family (Hambrock et al., 2004; Shibanuma et al., 1993). BM-40/SPARC/osteonectin proteins function as regulators of interactions of cells with the extracellular milieu during development, in response to injury, and in other settings (Bradshaw and Sage, 2001). However, Fstl1 lacks a key structural/functional characteristic of the BM-40/SPARC/osteonectin protein family in that its extracellular calcium-binding domain is nonfunctional (Hambrock et al., 2004) indicating Fstl1 may have distinct functional features. The presence of a follistatin-like domain module that lies between the Fstl1 signal sequence and the extracellular calcium-binding domain region renders Fstl1 distantly-related to the activin and bone morphogenetic protein (BMP) antagonist follistatin (Hambrock et al., 2004; Shibanuma et al., 1993). Follistatin binds and sequesters activin (Chen et al., 2006); however binding of Fstl1 with activin has not been demonstrated. Although there are three other genes that are "follistatin-like" (Fstl3, Fstl4, and Fstl5), these have only weak and limited sequence relationship with Fstl1. Fstl4 and Fstl5 are essentially uncharacterized. Fstl3 has been demonstrated to bind and neutralize TGF-β family ligands, including activin and myostatin (Sidis et al., 2006). Fstl3 null mice exhibit a set of metabolic phenotypes including enhanced insulin sensitivity and reduced visceral fat (Mukherjee et al., 2007).

Initial reports supported a putative tumor suppressor function for Fstl1. For example, Fstl1 transcript expression was decreased in a panel of cancer cells (Mashimo et al., 1997) and following oncogenic transformation of fibroblasts (Johnston et al., 2000; Shibanuma et al., 1993). Ectopic expression of Fstl1 was reported to diminish growth of cancer cells (Sumitomo et al., 2000) and to decrease invasiveness of oncogenically-transformed cells (Johnston et al., 2000). In the last several years, functional roles for Fstl1 have been described in respect to BMP4 signals in embryonic development (Esterberg et al., 2008), revascularization after ischemic injury (Liu et al., 2006; Ouchi et al., 2008), myocyte cell survival (Oshima et al., 2008), immunomodulation (Kawabata et al., 2004; Le Luduec et al., 2008), cytokine regulation (Clutter et al., 2009; Miyamae et al., 2006), and cancer (Chan et al., 2009; Reddy et al., 2008; Trojan et al., 2005).

Information on Fstl1 in adipogenesis is limited to that included in a single report on in *in vitro* vs. *in vivo* adipogenesis transcriptional profiles using high density microarrays. In this study by Friedman and coworkers, Fstl1 was among the set of genes categorized as both highly enriched in 3T3-L1 preadipocytes vs. 3T3-L1 adipocytes and in the preadipocyte-containing SVF vs. adipocytes purified from WAT *in vivo* (Soukas et al., 2001). To our knowledge, this observation was not further followed up in respect to Fstl1, nor have any other reports addressed the expression, regulation, and potential role of Fstl1 in the adipocyte lineage. Through differential hybridization screening of nylon DNA arrays we identified Fstl1 transcript to be highly expressed in preadipocytes and to dramatically decrease during adipogenesis, becoming nearly undetectable in cultures of differentiated adipocytes and we report on the further characterization the expression and regulation of Fstl1 in preadipocytes and in adipogenesis.

2. Results

2.1. *Identification of Fstl1 as a robust preadipocyte signature gene*

To identify and characterize distinct gene expression patterns that may define preadipocytes, we undertook DNA array analysis of ~5000 murine genes utilizing filter arrays. Differential screening was conducted *via* hybridization with reverse-transcribed cDNA probes prepared from 3T3-L1 preadipocyte or 3T3-L1 adipocyte total RNA. Fstl1 demonstrated a very high degree of differential transcript enrichment in preadipocytes vs. adipocytes. A portion of the arrays showing the differential signal for Fstl1 is shown in Fig. 1A. Fig. 1B depicts the structural features found in the 306 amino acid Fstl1 protein. These are an N-terminal signal sequence, a single follistatin-like domain module showing distant homology to follistatin, a region of homology with the extracellular calcium-binding domain of the BM40/SPARC/osteonectin family, and a von Willebrand type C homology region. To further assess expression of Fstl1 transcript during adipocyte differentiation, we examined a time course of *in vitro* adipocyte conversion of murine 3T3-L1 preadipocytes using Northern blot analysis. For 3T3-L1 differentiation, adipogenesis is induced by a standard 2-day treatment with Dex and Mix; a high degree of adipocyte conversion usually occurs by 5–7 days. As shown in Fig. 1C, downregulation of Fstl1 transcript is first noted at 2 days, prior to the removal of adipogenic inducing agents. It is further decreased at 3 days and is markedly reduced upon full differentiation to mature adipocytes at 5–7 days. Here, Pref-1 was utilized as a preadipocyte marker transcript and PPARγ and adipocyte fatty acid binding protein (aFABP) transcripts as adipocyte markers. As shown, the downregulation of Fstl1 transcript at 2 days is inversely related to the appearance of transcripts for these adipocyte-specific genes.

To examine expression of Fstl1 in very early 3T3-L1 adipogenesis, we conducted time course studies focusing on the first 2 days. Q-PCR analysis in Fig. 1D shows that during the first 1.5 days of adipogenic induction a transient increase of Fstl1 transcript is noted, peaking at ~2-fold at 0.25–0.5 day post-adipogenic induction. This is followed by a ~5-fold diminution of Fstl1 transcript expression between 1.5 and 2 days and a ~12-fold decrease of Fstl1 transcript by 7 days. While we have not investigated the function nor mechanism of

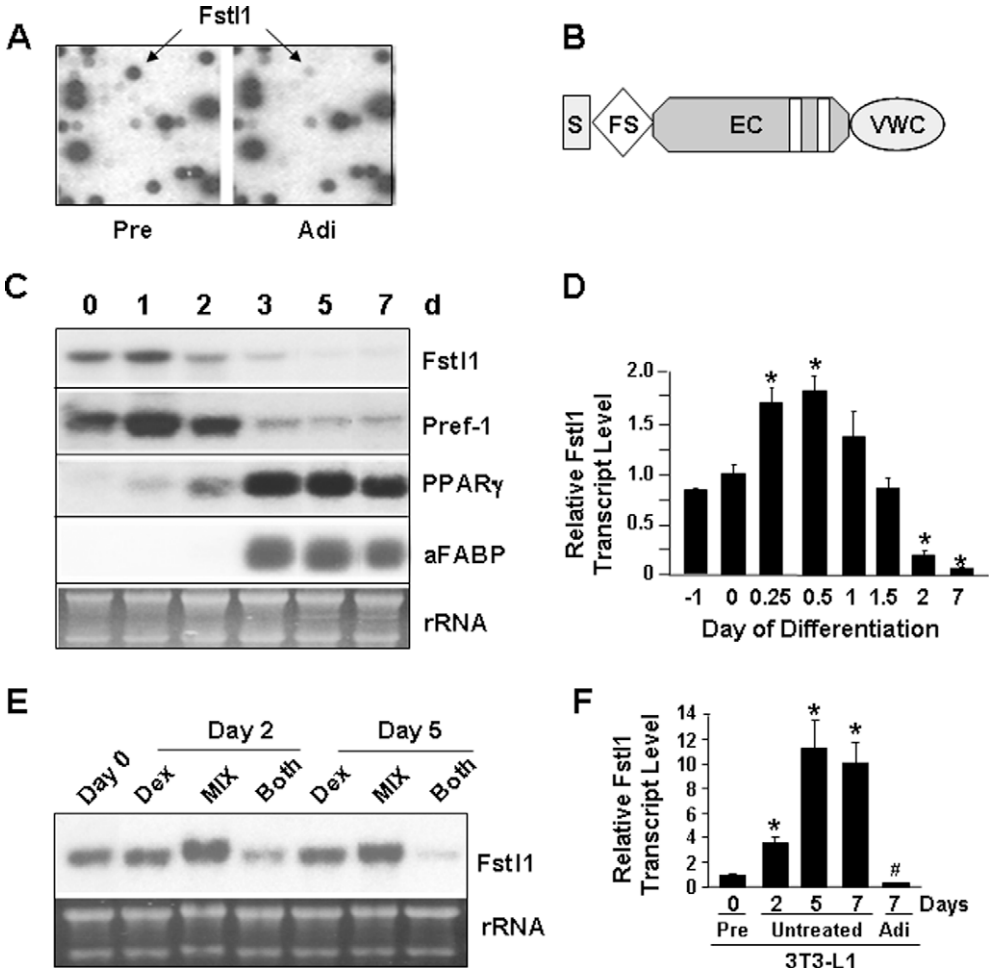

Fig. 1 – Fstl1 transcript expression during adipocyte conversion of 3T3-L1 preadipocytes. (A) Initial differential screening identification of Fstl1 as a novel gene enriched in 3T3-L1 preadipocytes vs. 3T3-L1 adipocytes. Shown is a portion of the DNA filter array indicating Fstl1 signal (arrows) in 3T3-L1 preadipocytes (Pre) and minimal signal in 3T3-L1 adipocytes (Adi). (B) Structural motifs of the Fstl1 protein. S, signal sequence; FS, follistatin-like module; EC, extracellular calcium-binding homology region with vertical bars indicating EF-hand motifs; VWC, von Willebrand type C homology region. (C) Regulation of Fstl1 transcript in 3T3-L1 adipogenesis. 3T3-L1 cells were harvested as preadipocytes before induction of adipogenesis (0) and at indicated time points in days (d) post-induction of adipocyte differentiation. Northern blot analysis was performed with murine Fstl1, Pref-1, PPARγ and aFABP cDNA probes. (D) Q-PCR analysis of Fstl1 transcript expression in 3T3-L1 adipogenesis. Signal was corrected against Gapdh level. *$p < 0.01$ compared to day 0 sample. Value in 0 day was set to 1. (E) Fstl1 transcript regulation by adipogenic cocktail components. Post-confluent 3T3-L1 preadipocytes were treated with 10% FBS supplemented with 1 μM Dex, 0.5 mM Mix, or both Mix and Dex for 48 h. Cultures were either harvested at 48 h or maintained through day 5 in 10% FBS and then harvested. Northern blot was performed using murine Fstl1 cDNA probe. For (C) and (E), the EtBr staining of rRNA is shown as a gel loading control. (F) Q-PCR analysis of Fstl1 transcript in post-confluent 3T3-L1 preadipocytes. Cells were harvested at 0 day, or at 7-day post-standard adipogenic induction (first and last lanes). Cells not treated for adipogenic induction (untreated) were harvested at indicated days following the 0 day time point. *,#$p < 0.001$ in regard to increased or decreased Fstl1 transcript level relative to 0 day, respectively.

the early transient increase in Fstl1 transcript, its increase is coincident with the clonal expansion phase of *in vitro* adipogenesis as post-confluent preadipocytes undergo several rapid rounds of the cell cycle (Tang et al., 2003a,b). We next examined the regulation of Fstl1 transcript in response to treatment of 3T3-L1 preadipocytes with the individual Dex and Mix components of the adipogenic cocktail. While the response of preadipocytes to Dex/Mix induced adipogenesis is likely complex and combinatorial, members of the C/EBP family of pro-adipogenic transcription factors were the first identified direct targets for Dex and Mix action (Cao et al., 1991; Umek et al., 1991; Yeh et al., 1995). Upon adipogenic induction, Dex stimulated C/EBPδ gene expression and MIX stimulated C/EBPβ expression (Cao et al., 1991; Umek et al., 1991; Yeh et al., 1995). These early responses feed into subsequent transcriptional cascades including upregulation of C/EBPα (Cao et al., 1991; Umek et al., 1991; Yeh et al., 1995). Downregulation of Fstl1 transcript requires the combined actions of the Dex/Mix adipogenic cocktail and is not regulated by either individual component (Fig. 1E).

Lastly we further confirmed that the decrease we observed in Fstl1 transcript level was dependent on the adipogenic program and not merely due to the consequence of prolonged in *vitro* culture of 3T3-L1 preadipocytes, irrespective of whether or not they were treated with adipogenic agents (Fig. 1F). For this, cultures of 3T3-L1 preadipocytes at 2-day post-confluence, the standard point for induction of adipogenesis, were either changed into Dex/Mix adipogenic media for 2 days or subject only to media change with standard growth media. RNA was analyzed from 0 day cultures and from preadipocyte cultures at 2, 5, and 7 days; these latter three samples had not been exposed to adipogenic treatment. We also analyzed RNA from cultures at 7-day post-adipogenic induction for comparison; these evidenced the typical morphology of mature lipid-filled adipocytes (data not shown). As is shown in Fig. 1F, we observed the anticipated downregulation of Fstl1 transcript in 7-day adipocytes vs. 0-day preadipocytes (first and last columns, respectively). However, contrary to this downregulation as cells undergo adipogenic conversion, we found an ~10-fold increase in Fstl1 transcript level in parallel cultures of untreated 3T3-L1 preadipocytes (i.e. those not subject to adipogenic hormone treatment) that were maintained in culture through 7 days. While we do not yet know what role this increase in Fstl1 transcript level may play in post-confluent 3T3-L1 preadipocytes, together our data clearly show that decreased levels of Fstl1 transcript are specific to the adipogenic differentiation program. It is not merely a result of prolonged maintenance of cultures of post-confluent, presumably growth-arrested, 3T3-L1 preadipocytes.

We extended study of differentiation-dependent downregulation of Fstl1 transcript to additional models of *in vitro* adipogenesis. The Northern blot in Fig. 2A shows marked downregulation of Fstl1 transcript also accompanies adipogenic conversion of ScAP-23 cells, a new white preadipocyte cell line generated in our laboratory from murine subcutaneous WAT (Kim et al., 2007a). Mammals also have brown adipose tissue (BAT) which serves primarily to dissipate energy as opposed to storing it (Lowell and Flier, 1997). Brown adipocytes are present in adult humans and have an emerging role in human metabolism and obesity (Cinti, 2006). Whereas it was initially thought that these brown adipocytes were found only interspersed within select other tissues, it has recently been reported that BAT can be present as a distinct tissue entity in some adult humans (Cypess et al., 2009). The Northern blot in Fig. 2B shows that, similar to white adipogenesis, downregulation of Fstl1 transcript level is also a hallmark of brown adipogenesis. This was examined using an established brown adipocyte cell line (WT-BAT) established by Kahn and coworkers from BAT of neonatal mice (Klein et al., 2002). The expression of the brown adipocyte-specific transcript uncoupling protein 1 (UCP1) at day 8 is a marker of brown adipocyte conversion. Fig. 2C is Q-PCR data for levels of Fstl1 transcript in preadipocytes and mature 7-day adipocytes in five *in vitro* models of adipogenesis. The Ad2.7AC cell line is derived from bone marrow stromal cells and is committed to an adipocyte lineage (Lecka-Czernik et al., 1999). The mBAP-9 preadipocyte cell line has recently been generated in this laboratory from BAT of 3-week-old mice (manuscript in preparation). Fstl1 is a differentiation-dependent downregulated transcript in each of these cell culture models of *in vitro* adipogenesis.

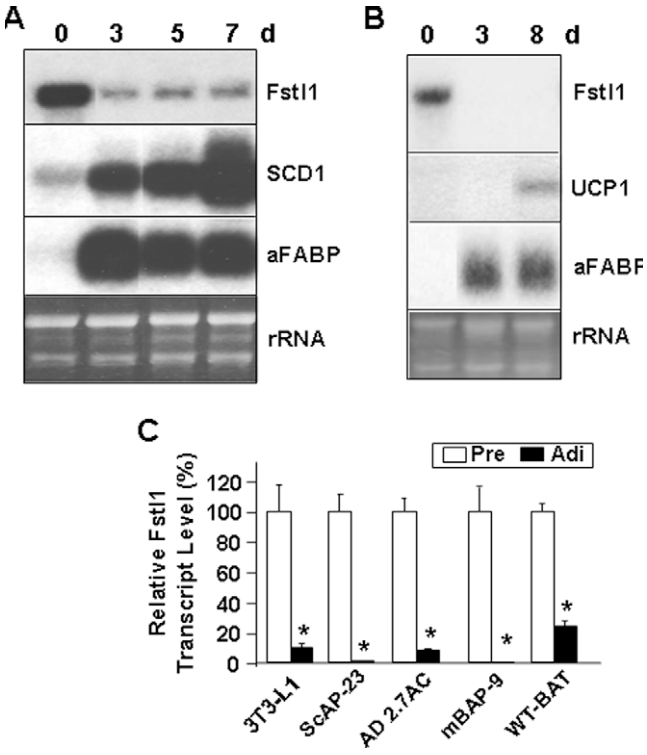

Fig. 2 – Fstl1 transcript expression is decreased in multiple *in vitro* models of white and brown *in vitro* adipogenesis. (A) Fstl1 downregulation during ScAP-23 differentiation. Cells were harvested as preadipocytes prior to induction of adipogenesis at day (d) 0 and at indicated days post-induction of adipogenic differentiation. Northern blot shows transcript level for Fstl1, SCD1 and aFABP. (B) Fstl1 transcript expression is decreased in brown adipogenesis. WT-BAT preadipocytes were harvested at confluence (0 d) and adipocytes harvested at day 3 and day 8 post-induction of differentiation. Northern blot analysis was performed with murine Fstl1, UCP1, and aFABP probes. (A, B) EtBr staining of rRNA is shown as a gel loading control. (C) Q-PCR analysis of Fstl1 transcript levels in preadipocytes (Pre) and adipocytes (Adi) of 3T3-L1, ScAP-23, AD2.7AC, mBAP-9, WT-BAT cell lines. Value in the respective preadipocyte sample was set to 100% and *$p < 0.001$ for Adi vs. Pre.

2.2. *Fstl1 transcript expression in WAT is restricted to the preadipocyte-containing stromal-vascular fraction*

A microarray analysis by Soukas and coworkers revealed distinct transcriptional profiles for 3T3-L1 *in vitro* vs. *in vivo* murine adipogenesis (Soukas et al., 2001); in that case comparison of the preadipocyte-containing stromal-vascular fraction (SVF) and adipocyte fraction of WAT served as a proxy for *in vivo* white adipogenesis. Soukas and coworkers reported that a large number of genes are highly differentially expressed for *in vitro* vs. *in vivo* sources (Soukas et al., 2001). Of interest, Fstl1 was among the various genes listed in their microarray data to be enriched in preadipocytes vs.

adipocytes both *in vitro* and *in vivo*; suggestive of importance in the adipocyte lineage. To investigate Fstl1 transcript regulation during adipogenesis in a model that represents the process of adipogenesis *in vivo* we used collagenase digestion of murine WAT to prepare purified adipocytes and the preadipocyte-containing SVF. The Northern blot in Fig. 3A illustrates that Fstl1 transcript was not detected in the adipocyte fraction of murine SC WAT, a cell population that does not contain preadipocytes, but is readily detected in the preadipocyte-containing SVF. For this study, stearoyl-CoA desaturase-1 (SCD1) small adipocyte factor 1 (SMAF1) and resistin (Retn) transcripts were used as adipocyte markers. While our data does not exclude the possibility that other SVF cell types may also be a source of Fst1l expression within WAT, we have found that the SVF and each of the preadipocyte cell lines examined in Fig. 2C express from ~500 to ~3000 times higher levels ($p < 0.01$) of Fstl1 transcript level than do cells of the murine macrophage line RAW264.7 (data not shown). This suggests that macrophages within WAT are likely not a source of Fstl1 transcript. This view is also supported by the fact that we do not observe any consistent degree of increase in Fstl1 transcript level in *obese* (*ob*/*ob*) WAT compared with that for wildtype mice (data not shown). Obese mice evidence dramatically enhanced macrophage numbers within their WAT, which results in the detection of markedly increased levels for macrophage-expressed transcripts therein (Cancello et al., 2006; Kamei et al., 2006; Permana et al., 2006; Weisberg et al., 2003). If macrophages made a significant contribution to WAT level of Fstl1 transcript expression, we would expect to observe a dramatically increased Fstl1 transcript level in obese WAT.

It is well-established that increased intra-abdominal WAT is linked to a higher risk of diabetes and cardiovascular disease compared to subcutaneous adipose tissue (SC), suggestive of functional heterogeneity of adipocytes (Gesta et al., 2007). We used Q-PCR, Fig. 3B, to compare Fstl1 transcript expression in intra-abdominal epididymal (EP) WAT and in subcutaneous (SC) WAT. As expected, for SC and EP WAT, Fstl1 transcript is enriched over 50-fold in the SVF vs. the adipocyte fraction. Additionally, the Fstl1 transcript level in EP SVF is

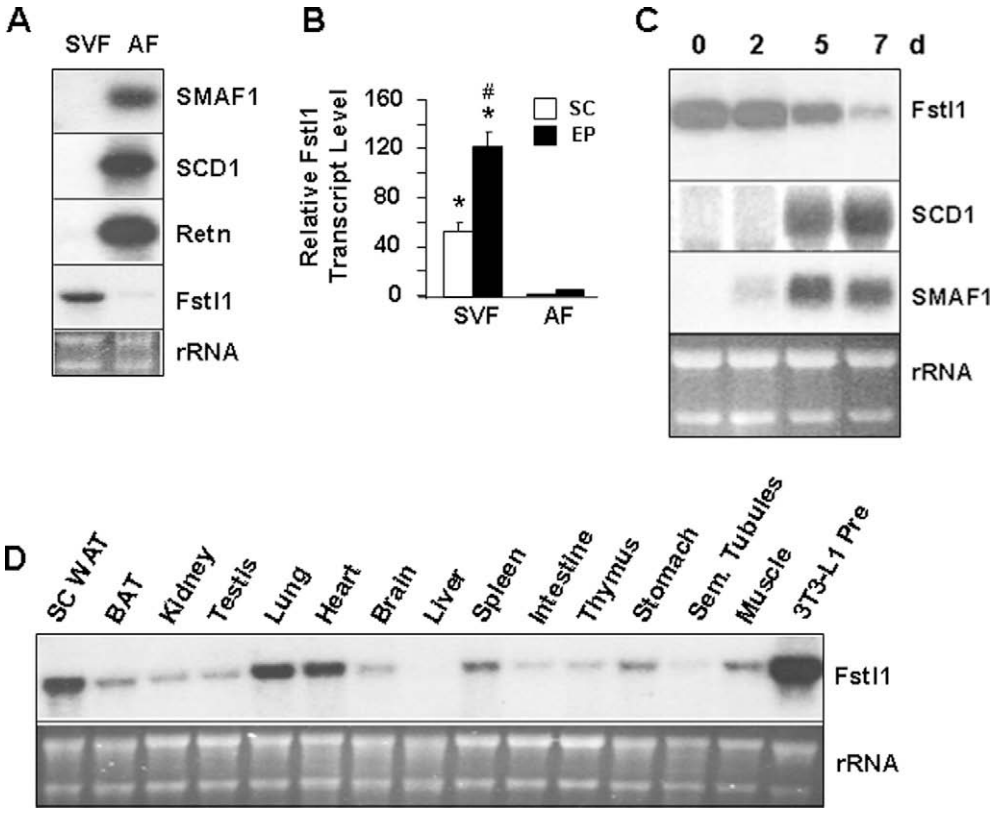

Fig. 3 – Fstl1 transcript expression in cellular fractions of WAT and in adipogenesis of primary preadipocytes. (A) Fstl1 transcript in stromal-vascular fraction (SVF) and adipocyte fraction (AF) of murine WAT. Northern blot shows transcript level for SMAF1, SCD1, Retn and Fstl1. (B) Q- PCR assessment of Fstl1 transcript level in cell fractions of WAT. cDNA derived from SVF or adipocyte fraction (AF) of subcutaneous (SC) or epididymal (EP) WAT was used for Q-PCR analysis of Fstl1 transcript. *Note*. The third column represents SC tissue but the white color of the bar cannot be discerned due to low column height. The level in SC AF was set to 1. $^{*}p < 0.001$ for SC SVF vs. SC AF and for EP SVF vs. EP AF. $^{\#}p < 0.001$ for EP SVF vs. SC SVF. (C) Fstl1 transcript downregulation during adipogenic conversion of rat primary white preadipocytes. RNA was collected from preadipocytes (0) and at indicated times post-induction of adipocyte differentiation and analyzed by Northern blot using murine Fstl1, SCD1 and SMAF1 cDNA probes. (D) Fstl1 transcript level in a panel of adult murine tissues. Five micrograms of total RNA from indicated tissues were analyzed by Northern blot using murine Fstl1 cDNA probe. For (A, C and D), EtBr staining of rRNA is shown as a gel loading control.

approximately 2-fold higher than that in SC SVF, indicating Fstl1 appears to demonstrate a degree of depot-dependent differential expression (Fig. 3B). We next examined regulation of Fstl1 transcript following culture of the preadipocyte-containing SVF cells harvested from rat WAT and during differentiation of these primary white preadipocytes to adipocytes, Fig. 3C. We utilized rat cells for this study as we typically observe a more uniform and a higher percentage of adipocyte differentiation with primary cultures of rat SVF cells compared with murine SVF cells. Dramatic downregulation of Fstl1 transcript occurred as a result of differentiation of primary white preadipocytes to adipocytes (Fig. 3C). As was noted for the established preadipocyte cell lines, presented in Figs. 1 and 2, the primary cell data indicates that downregulation of Fstl1 transcript also inversely correlates with emergence of the adipocyte phenotype, indicated by expression of the adipocyte marker transcripts SCD1 and SMAF1. The Northern blot in Fig. 3D shows that SC WAT is a primary site of Fstl1 transcript expression, with prominent expression also noted for lung and heart. Markedly lower levels of Fstl1 transcript were detected most other tissues examined except for liver, which appears negative. While our studies are the first to report Fstl1 transcript expression levels in WAT, data on other murine tissues shown in Fig. 3D is largely consistent with published reports (Adams et al., 2007; Liu et al., 2006; Mashimo et al., 1997).

2.3. *Expression and regulation of Fstl1 protein in adipogenesis*

To begin studies on the Fstl1 protein, we first characterized a commercially available Fstl1 antibody utilizing transfection of an HA-tagged Fstl1 expression construct into COS cells, followed by analysis of lysate and culture media by Western blot. The top panel of Fig. 4A indicates successful detection of the Fstl1-HA protein in media and cells using anti-HA antibody, consistent with the secreted nature of Fstl1 protein. The lower panel of Fig. 4A indicates that anti-mouse Fstl1 antibody readily detects ectopically expressed Fstl1-HA protein in both COS cell lysates and culture media. The molecular mass found for secreted Fstl1 in media appears larger than that found in cell lysates, consistent with a previous report on glycosylation of Fstl1 (Hambrock et al., 2004). No background or cross-reactive species are evident for vector-only transfectants, confirming the utility of the Fstl1 antibody for Western blot analysis. To determine Fstl1 protein expression during adipogenesis, 3T3-L1 cell lysates and culture media were collected prior to induction of adipogenesis as confluent preadipocytes (0 d), or at daily times post-induction of differentiation. An anti-PPARγ antibody was employed as a marker for adipocyte differentiation. Endogenous Fstl1 protein level in 3T3-L1 cell lysates was downregulated at 1-day post-treatment with adipogenic agents and markedly decreased levels were noted at 5–6 days. Fstl1 protein was all but absent by 7- to 8-days post-adipogenic induction, Fig. 4B. Fig. 4C illustrates that Fstl1 protein is secreted into culture media by 3T3-L1 preadipocytes, defining Fstl1 as a new “preadipokine”. Levels of Fstl1 protein secreted into media declined dramatically by 3 days. For both Fig. 4B and C, we chose to present blot data of a longer film exposure time

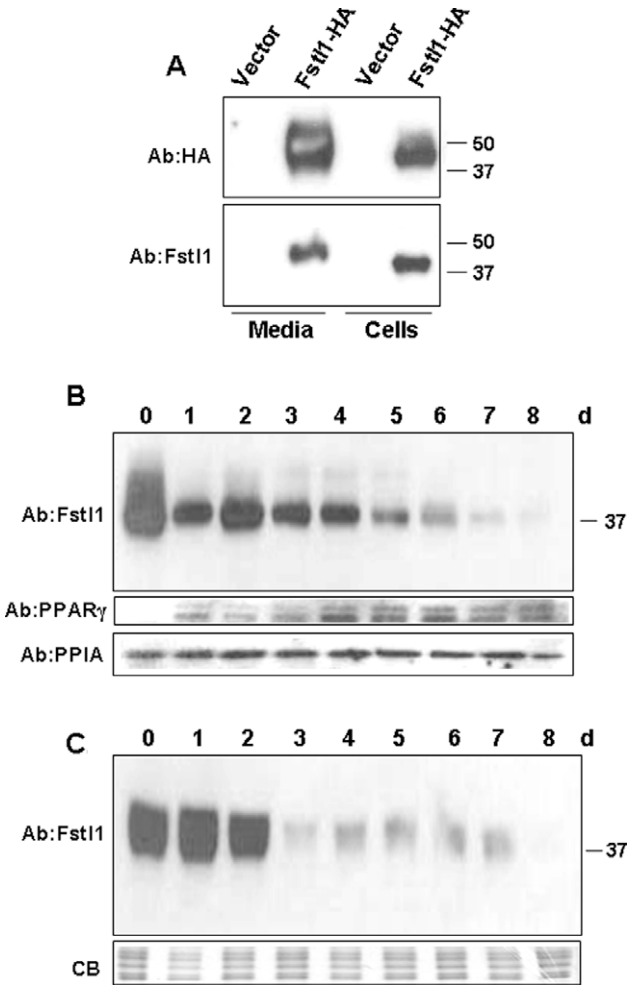

Fig. 4 – Expression and secretion of Fstl1 protein during 3T3-L1 adipogenesis. (A) Assessment of Fstl1 antibody. Vector only or an Fstl1-HA expression construct was transiently transfected into COS cells. Culture media and cell lysates were analyzed by Western blot using anti-HA (top panel) or anti-Fstl1 (lower panel) primary antibodies. (B, C) Fstl1 downregulation in 3T3-L1 cell lysates (B) and culture media (C) during adipocyte differentiation. Cells and culture media were harvested from confluent 3T3-L1 preadipocytes prior to induction of adipogenesis (day 0) or daily post-induction of differentiation. Western blot was performed using anti-Fstl1 primary antibody. For (B – lower two panels), the membrane was re-processed using anti-PPARγ primary antibody as a differentiation marker and anti-PPIA (peptidylprolyl isomerase A) primary antibody as a loading control. For (C – lower panel), the gel was stained with Coomassie Blue (CB) to assess evenness of sample loading. For Fstl1 blots in (B, C), a longer exposure is presented to best illustrate signal at the later time points.

in order to be fully informative in respect to the lower levels of Fstl1 protein expressed at the later time points. As a result earlier time points appear somewhat overexposed. To determine protein half-life for secreted Fstl1, 3T3-L1 preadipocytes were cultured with cycloheximide. Media was collected at subsequent time points and assessed for endogenous Fstl1

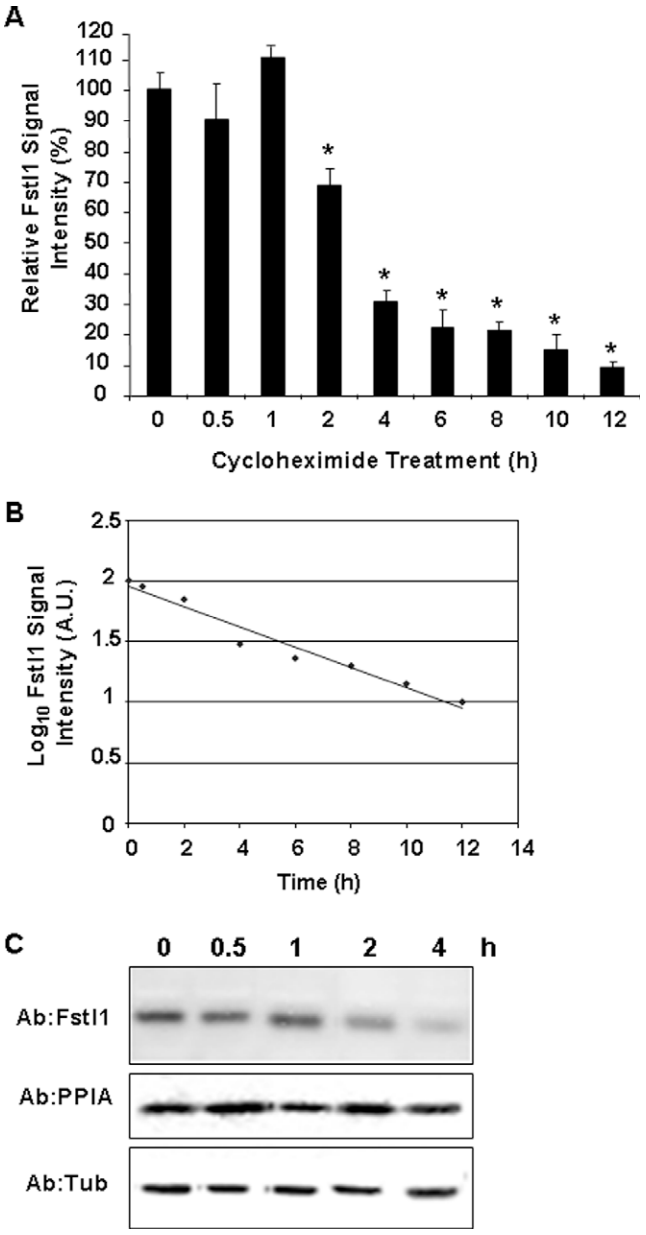

Fig. 5 – Determination of protein half-life for endogenous preadipocyte-secreted Fstl1. 3T3-L1 preadipocytes were treated with 5 μg/ml of cycloheximide and harvested at indicated time points in hours (h) post-treatment. (A) Quantitation of Western blot signal for preadipocyte-secreted Fstl1 in triplicate time point samples. (B) Semi-log plot of decrease of preadipocyte-secreted Fstl1 protein level over time in hours (h). (C) Western blot for preadipocyte-secreted Fstl1 protein in representative of samples for the 0–4 h time points. β-Tubulin and PPIA are shown as controls. A shorter exposure is presented in order to best visualize Fstl1 signals at each intermediate time point.

protein by Western blot analysis using anti-Fstl1 antibody. The graph in Fig. 5A shows quantitation of Western blot signals and a semi-log plot of the results is presented in Fig. 5B. These data indicate that the protein half-life of preadipocyte-secreted Fstl1 is ~3 h. A representative Western blot for Fstl1 protein for the 0–4 h time points of the cycloheximide study are shown in Fig. 5C; a minimal exposure time for digital image acquisition was used such that signal differences at each of the time points are maximally evident. Fstl1 protein has been demonstrated to be a target for MMP-2 mediated proteolytic degradation to generate multiple smaller protein species (Dean et al., 2007) and active MMP-2 is present in 3T3-L1 preadipocytes (Croissandeau et al., 2002). However, in our studies to date of preadipocyte-secreted Fstl1, we have not observed shorter Fstl1 species indicative of proteolysis.

2.4. *Re-emergence of Fstl1 expression tracks with TNFα-induced adipocyte de-differentiation*

It is well known that TNFα is a contributing cause of the insulin resistance seen in obesity and obesity-linked diabetes (Hotamisligil, 2006). Genome-scale transcriptional profiling has revealed that TNFα treatment of 3T3-L1 adipocytes results in suppression of a large number of adipocyte-specific genes while concomitantly activating many preadipocyte genes (Ruan et al., 2002a). TNFα is also a major regulator of WAT gene expression *in vivo* (Ruan and Lodish, 2003; Ruan et al., 2002b). Western blot analysis in Fig. 6A and B indicates TNFα treatment of 3T3-L1 adipocytes results in a marked upregulation of Fstl1 protein level both in cell lysates and in media. The Northern blot in Fig. 6C shows that treatment of 3T3-L1 adipocytes with TNFα results in re-expression of Fstl1 transcript over the 36 h time period examined. Decreased SMAF1 and PPARγ transcript expression indicate de-differentiation of adipocytes, as anticipated. These observations of re-emergence of Fstl1 expression upon TNFα-mediated adipocyte de-differentiation indicate that Fstl1 transcript and protein expression closely tracks with, and is inversely related to, the adipocyte phenotype. As was previously reported by others (Xing et al., 1997), transcript for the preadipocyte gene Pref-1 remains largely constant with TNFα treatment.

2.5. *Preadipocyte expression of Fstl1 is decreased by 5-Aza-CR*

To investigate signals that may regulate Fstl1 transcript expression, 3T3-L1 preadipocytes were individually treated with 18 hormones and other agents; the majority of these have roles in adipogenesis and/or adipocyte function. Fig. 7A shows Northern blot analysis of 3T3-L1 preadipocytes following 24 h agent treatment. Of all the agents tested, 5-aza-CR was observed to have the most dramatic effect on modulation of Fstl1 transcript level. The incorporation of 5-aza-CR, an analogue of cytosine, into DNA leads to inhibition of methyltransferase activity to result in DNA demethylation. Demethlyation of a gene is nearly exclusively reported to correlate with its activation (Christman, 2002; Jones, 1985). The decrease in Fstl1 transcript level by 5-aza-CR was confirmed on triplicate samples presented in Fig. 7B Northern blot. To quantitate these effects we used Q-PCR; this was assessed employing three separate internal standards, Gapdh, 36B4 and 18S (Fig. 7C). We find that, on average, 5-aza-CR resulted in an 89% decrease in Fstl1 transcript level in 3T3-L1 preadipocytes. Fig. 7D shows that protein level of Fstl1 in media of

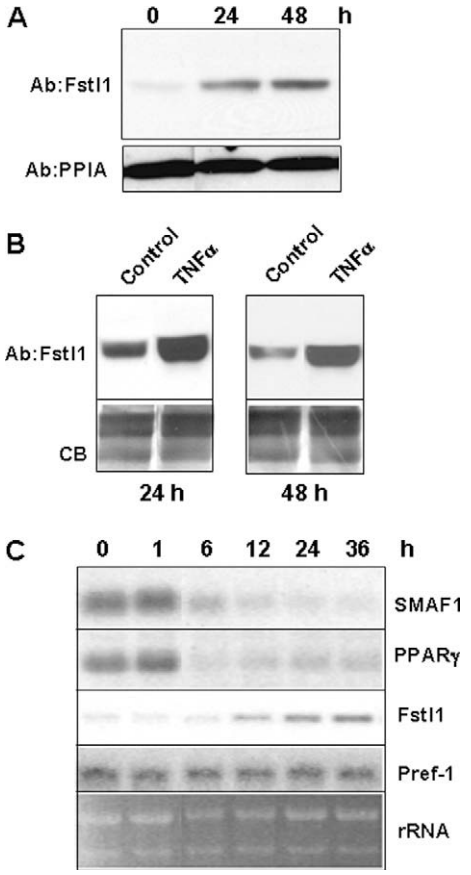

Fig. 6 – Regulation of Fstl1 protein and transcript expression by TNFα. (A, B) Fstl1 protein level in cell lysates (A) and in media (B) of 3T3-L1 adipocytes (time 0 and control, respectively) following treatment with 10 ng/ml TNFα for 24 h or 48 h. For (A), samples were processed for Western blot using either Fstl1 antibody or for loading control, anti-PPIA antibody. For (B), the gel was stained with Coomassie Blue (CB) to assess even sample loading. (C) Fstl1 transcript level in 3T3-L1 adipocytes during treatment with TNFα. 3T3-L1 adipocytes were treated with 10 ng/ml TNFα and RNA samples were collected prior to treatment (0) and at indicated times in hours (h) thereafter. Northern blot was performed using murine SMAF1, PPARγ, Fstl1 and Pref-1 cDNA probes. EtBr staining for rRNA is shown as a gel loading control.

3T3-L1 preadipocytes is markedly decreased after a 24 treatment with 5-aza-CR. We also examined the time course of Fstl1 transcript downregulation by 5-aza-CR in 3T3-L1 preadipocytes. Fig. 7E indicates that Fst1l transcript levels are decreased by 6 h of treatment and further decreased at 12–48 h. The transcript level of a control transcript 36B4 remained relatively unchanged through 48 h.

It has been demonstrated that for cultures of Swiss 3T3 and C3H10T1/2 mouse cells treated with 5-aza-CR, three mesenchymal-derived phenotypes, characterized as muscle cells, adipocytes and chondrocytes emerge (Pinney and Emerson, 1989; Taylor and Jones, 1979). Based on regulation of Fstl1 expression by 5-aza-CR, we hypothesize that Fstl1 may be involved in cell fate determination in the preadipocyte/mesenchymal lineage. To investigate whether the phenomenon of transcript downregulation by 5-aza-CR might be a common theme for genes that are specifically decreased during preadipocyte to adipocyte conversion, we carried out additional transcript analysis using Q-PCR. We have previously reported that downregulation of a subset of preadipocyte genes, which includes Lox, Thbs2, Ctnnb1, Slc7a5, Aurkb and Mtap1 occurs in both 3T3-L1 and ScAP-23 adipogenesis (Kim et al., 2007a). Other studies have indicated the specific expression/enrichment of Col1A1 (Weiner et al., 1989; Yi et al., 2001), Sca-1/Ly6a (Rodeheffer et al., 2008), Pref-1(Smas et al., 1997; Smas and Sul, 1993), and GATA-3 (Tong et al., 2000) in adipogenic precursors. Fig. 8A–E reveals that transcript levels for a subset of the genes examined, namely Col1A1, Sca-1/Ly6a, Pref-1, Lox and Thbs2 are also downregulated by 5-aza-CR treatment of 3T3-L1 preadipocytes. Several of these genes (Col1A1, Pref-1, Thbs2), encode secreted/soluble factors (Bornstein et al., 2000; Smas et al., 1997; Weiner et al., 1989; Yi et al., 2001). Lox is known to be involved in extracellular matrix production (Lucero and Kagan, 2006), and Sca-1/Ly6a is a surface antigen (Rodeheffer et al., 2008). Transcript levels for the five other genes examined are very marginally increased (Fig. 8F–J).

2.6. *Functional analysis of the Fstl1 promoter region*

To date, there is no information regarding the transcriptional control of the Fstl1 gene promoter in any cell type, and very little is generally known on mechanisms of gene downregulation of preadipocyte-expressed genes during adipogenesis. To begin to characterize regulation of the Fstl1 promoter, two portions of the 5′ flanking regulatory region of the murine Fstl1 gene, encompassing −791/+60 and −3922/+22 bp, were used to generate luciferase reporter constructs, Fig. 9A. 3T3-L1 preadipocytes were transfected with these Fstl1 luciferase reporter constructs or empty pGL2-basic vector. As shown in the graph of Fig. 9B, luciferase activities of the Fstl1 luciferase promoter constructs are markedly elevated compared to pGL2-basic negative control.

We next explored possible mechanisms underlying differentiation-dependent downregulation of Fstl1 gene transcription. Unfortunately, attempts to compare Fstl1 promoter activity in 3T3-L1 preadipocytes vs. 3T3-L1 adipocytes were not fruitful due to inherent and well-known challenges of efficiently transfecting mature adipocytes. We also attempted to study differentiation-dependent transcriptional signals by assessing luciferase activity in 3T3-L1 stably transfected pooled populations of Fstl1 luciferase promoter constructs before and following their differentiation to adipocytes. We encountered a high degree of variation in such studies. We ascribe this to a reduced capacity 3T3-L1 preadipocytes to undergo effective adipocyte differentiation due to increased cell passaging needed in such studies, and perhaps also to integration site effects. We therefore turned to bioinformatic analysis of the sequence contained within the 791 bp fragment of the Fstl1 promoter to identify putative regulatory sites at which repressive transcription factors with a role in adipogenesis might function. Sequence analysis with MatInspector software identified four consensus inverted

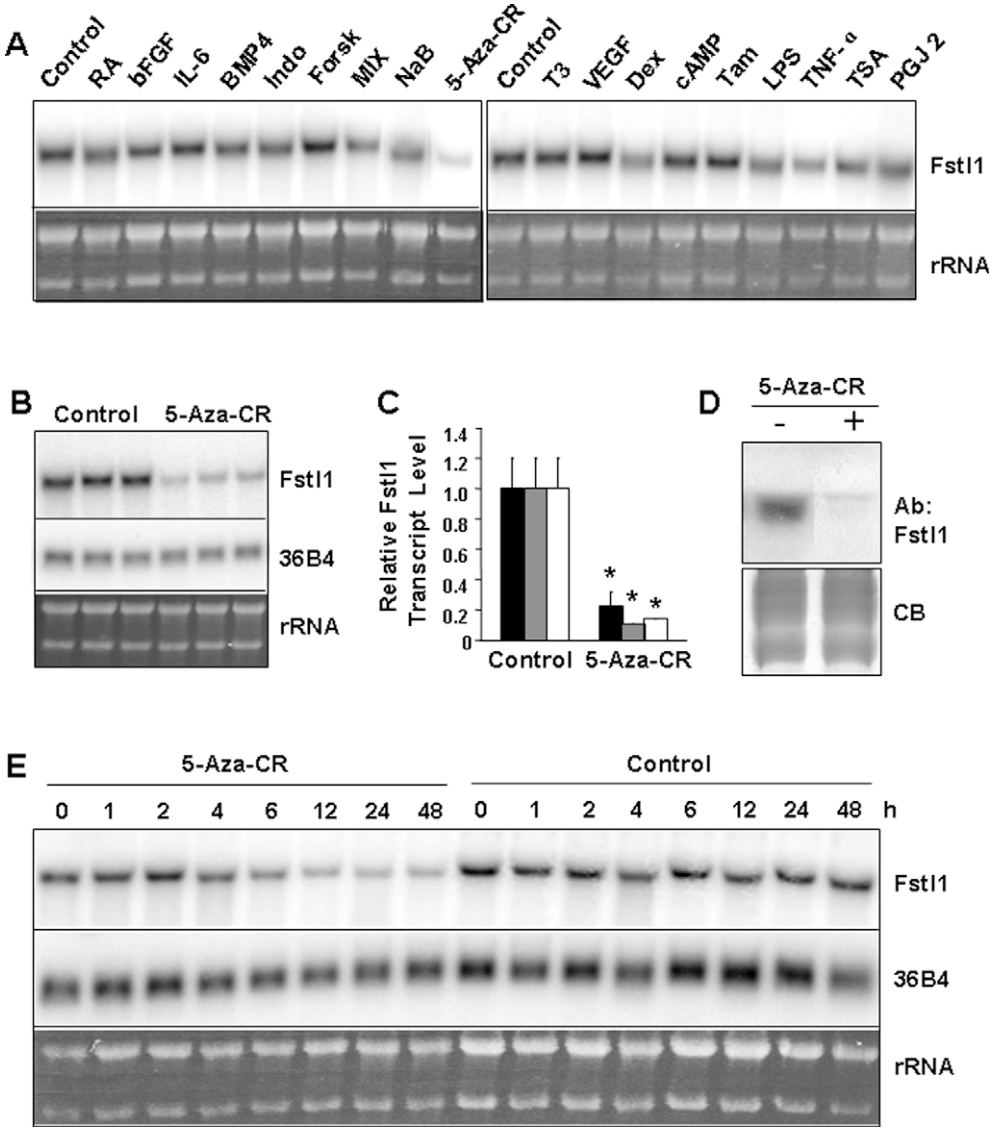

Fig. 7 – Levels of Fstl transcript and protein are decreased in 3T3-L1 preadipocytes by 5-Aza-CR treatment. (A) Confluent 3T3-L1 preadipocytes were either untreated (control) or treated with the indicated agents for 24 h. RNA samples were collected and analyzed by Northern blot using murine Fstl1 cDNA probe. The abbreviations used are: RA, retinoic acid; b-FGF, basic fibroblast growth factor; IL-6, interleukin-6; BMP4, bone morphogenetic protein 4; Indo, indomethicin; Forsk, forskolin; Mix, methylisobutylxanthine; NaB, sodium butyrate; 5-aza-CR, 5-aza-cytidine; T3, triiodothyronine; VEGF, vascular endothelial cell growth factor; Dex, dexamethasone; cAMP, cyclic adenosine monophosphate; Tam, tamoxifen; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; TSA, trichostatin A; PGJ2, prostaglandin J2. (B) Triplicate samples for control untreated or 5-aza-CR treated 3T3-L1 preadipocytes. Northern blot was performed using murine Fstl1 cDNA probe. (C) Q- PCR assessment of Fstl1 transcript level in control and 5-aza-CR treated 3T3-L1 preadipocytes. Fstl1 transcript signal was corrected against three internal standards (Gapdh, black bar; 36B4, gray bar; and 18S, white bar). The transcript level for control samples was set to a value of 1. *$p < 0.001$ for treated samples vs. respective controls. (D) Secreted Fstl1 protein expression by 3T3-L1 preadipocytes is decreased by 5-aza-CR treatment. 3T3-L1 preadipocytes were treated with 1 mM 5-aza-CR or untreated (control) for 24 h. Culture media was collected for Western blot analysis by using anti-Fstl1 antibody. The gel was stained with Coomassie Blue (CB) to assess evenness of sample loading. (E) Regulation of Fstl1 transcript expression during 5-aza-CR treatment of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were treated with 1 mM 5-aza-CR or untreated (control) for indicated time in hours (h). Northern blot was performed using a murine Fstl1 probe or a 36B4 probe as control. For (A, B and E), EtBr staining of rRNA is shown as a gel loading control.

Kruppel-like factor (KLF) binding sites. Three of these sites are closely grouped at the −280 to −350 bp region and a fourth is located between −70 and −86 (Fig. 9C and D).

KLFs are DNA-binding transcriptional regulators containing Cys2/His2 zinc fingers that play diverse roles during differentiation and development (Black et al., 2001; Dang et al.,

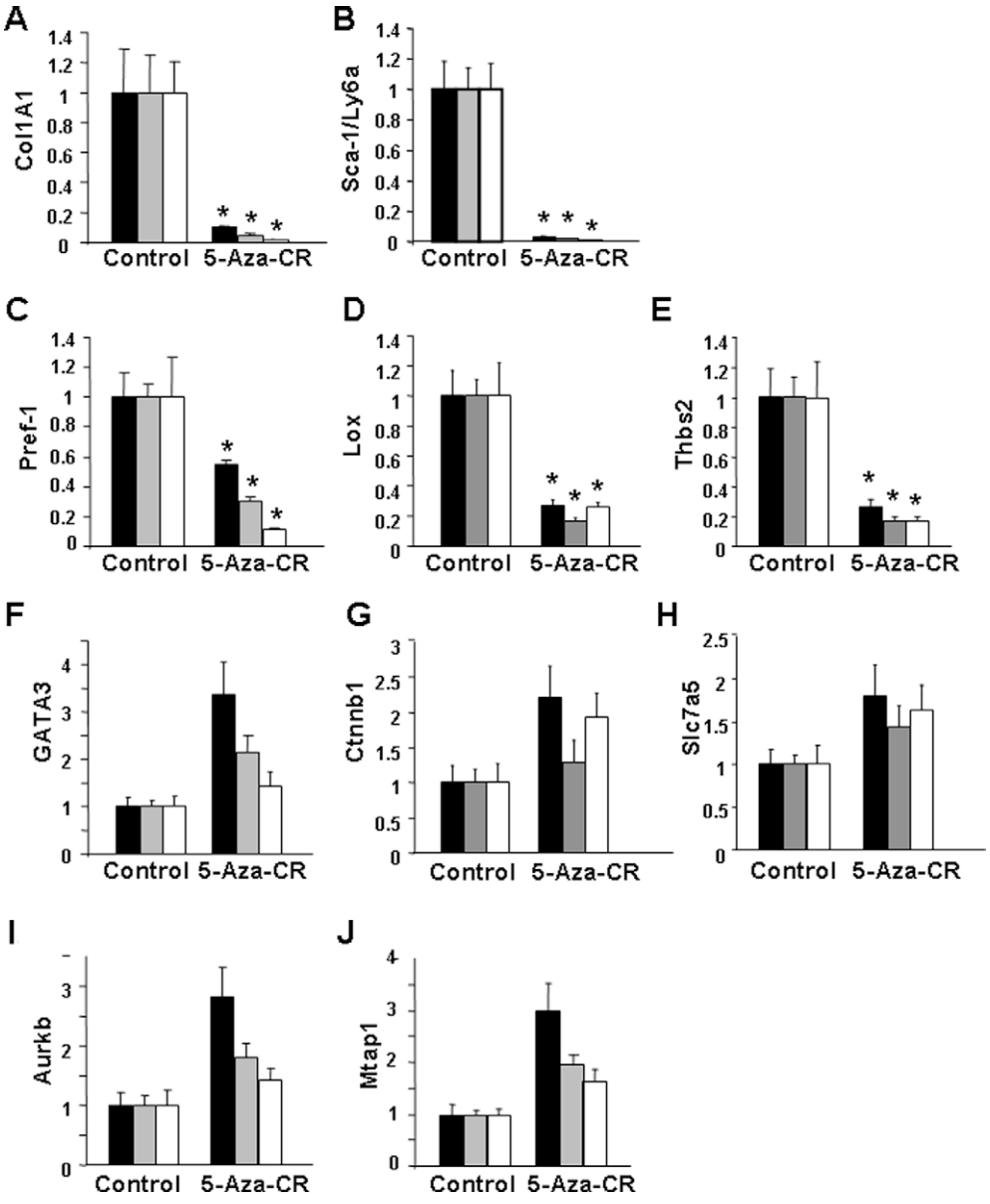

Fig. 8 – Assessment of effects of 5-aza-CR treatment of 3T3-L1 preadipocytes on transcript levels of 10 preadipocyte-selective genes. Q-PCR assessment of relative transcript level in untreated (control) and 5-aza-CR treated 3T3-L1 preadipocytes. Signal for each indicated transcript was corrected against three internal standards (Gapdh, black bar; 36B4, gray bar; and 18S, white bar). The transcript level for control samples was set to a value of 1. *Significant decrease ($p < 0.005$) for treated samples vs. their respective controls.

2000). Studies over the last several years have indicated a regulatory role for several KLFs in adipogenesis (Banerjee et al., 2003; Birsoy et al., 2008; Li et al., 2005; Mori et al., 2005; Oishi et al., 2005; Wu et al., 2005). A number of KLFs, such as KLF15, can function in both transcriptional activation and transcriptional repression in an apparently context-dependent manner. KLF15 expression is upregulated during adipocyte differentiation (Mori et al., 2005), which we also find (right panel of Fig. 9C), and KLF15 and C/EBPα act synergistically to increase the activity of the PPARγ gene promoter in 3T3-L1 (Mori et al., 2005). Overexpression of KLF15 induces adipocyte maturation and activates expression of the gene for the insulin-responsive glucose transporter, GLUT4, in adipocytes (Gray et al., 2002). On the other hand, KLF15 can also function in transcriptional repression; two genes transcriptionally repressed by KLF15 in adipocytes were recently identified (Nagare et al., 2009). We hypothesized that KLF15 might play a role in repression of Fstl1 gene expression in adipogenesis. We determined transcriptional regulation of the Fstl1 promoter by KLF15 employing co-transfection of Fstl1 luciferase constructs and a KLF15 expression construct in 3T3-L1 preadipocytes. In addition to the −791/+60 Fstl1 reporter construct, we generated a −350/+60 and a −280/+60 construct, the latter containing a single putative KLF15 binding site (Fig. 9C and D). As illustrated in Fig. 9E, relative luciferase activities of all three promoter constructs, −280/+60 LUC, −350/+60 LUC and

−791/+60 LUC are decreased by KLF15. These data suggest that differential expression of Fstl1 transcript in preadipocytes vs. adipocytes may involve transcriptional repression by KLF15.

3. Discussion

Our data clearly demonstrate that Fstl1 is expressed in preadipocytes and that dramatic downregulation of Fstl1 transcript and protein level is a robust hallmark of adipogenesis. Furthermore, Fstl1 transcript is re-expressed upon TNFα treatment of 3T3-L1 adipocytes. TNFα has been recognized as a link between obesity and insulin resistance and treatment of mature 3T3-L1 adipocytes with TNFα results in a degree of morphological, biochemical, and transcriptional de-differentiation to a preadipocyte-like phenotype (Ruan et al., 2002a,b; Ruan and Lodish, 2003). Fstl1 evidences a tight link to the preadipocyte phenotype as its expression closely tracks with TNFα-induced 3T3-L1 adipocyte de-differentiation. We find that Fstl1 is highly expressed in the media of cultured 3T3-L1 preadipocytes, typically detecting robust Fstl1 protein signal on Western blots using 5 μl or less of straight unconcentrated culture media. If Fstl1 has effects on adipogenic differentiation, or in regard to adipose tissue function, we postulate that it would most likely exert these as an autocrine or paracrine signal and perhaps through effects on cell matrix. Whether a specific receptor molecule exists for Fstl1 is not known nor have candidates been reported. Fstl1 was initially discovered as a TGF-β1-stimulated transcript in osteoblasts (Shibanuma et al., 1993). TGF-β1 has significant effects on ECM-related proteins through the induction of proteinase inhibitors and the suppression of proteinases that degrade matrix proteins (Shibanuma et al., 1993). *In vitro* and *in vivo* studies have shown that matrix remodeling is essential for adipogenesis and to also play key regulatory roles in WAT expansion in obesity (Lilla et al., 2002; Rupnick et al., 2002). Members of the BM-40/osteonectin/SPARC protein family have been demonstrated to contribute to the organization of the extracellular matrix *via* growth factor modulation, cell adhesion inhibition, proteinase inhibition and other effects (Bradshaw and Sage, 2001). It is possible that Fstl1 may affect ECM remodeling during adipogenesis. To date our attempts at using shRNA-mediated knockdown to address function of Fstl1 in the adipocyte lineage have been ineffective in achieving significant reduction of Fstl1 protein level in 3T3-L1

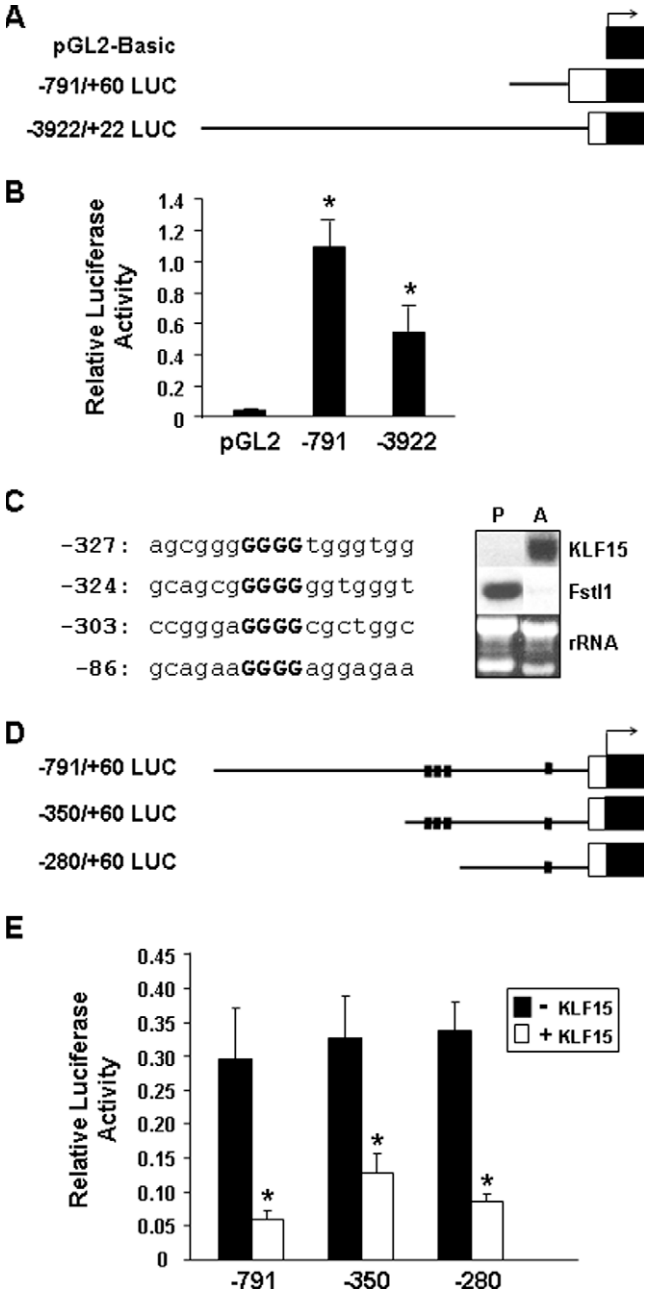

Fig. 9 – Promoter activity and KLF15 regulation of the murine Fstl1 5′ flanking region. (A) Schematic diagram of Fstl1 luciferase constructs. The solid line represents the Fstl1 promoter region, the white box the 5′ untranslated region of Fstl1, and the black box the luciferase reporter gene. B. Promoter activity of Fstl1 5′ flanking region in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were transfected with the indicated luciferase reporter constructs (−791 or −3922) or empty pGL2-Basic vector (pGL2). An * indicates $p < 0.001$ compared to empty pGL2-Basic vector. (C) Left panel: Sequence of putative KLF15 sites in the Fstl1 5′ flanking region. The consensus core motif of the inverted KLF sites are in bold. Numbers at left refer to the most 5′ position of the sequence relative to the Fstl1 transcription start site. Right panel: Northern blot analysis of KLF15 and Fstl1 transcripts in 3T3-L1 preadipocytes (P) and 7-day adipocytes (A), with rRNA shown as a loading control. (D) Schematic diagram of Fstl1 luciferase constructs containing putative KLF15 binding sites. The diagram key is as for A, above. The small black squares indicate putative KLF15 binding sites. (E) Regulation of Fstl1 promoter by KLF15 in preadipocytes. 3T3-L1 preadipocytes were transfected with luciferase reporter constructs containing 791, 350 or 280 bp of the Fstl1 gene 5′ flanking region with or without co-transfection of a KLF15 expression construct. *$p < 0.01$ for (+) KLF15 co-transfection vs. (−) KLF co-transfection. Data represent mean ± S.D. from a minimum of triplicate transfections.

preadipocytes. This may be attributable to the very high level of Fstl1 transcript and protein expressed by these cells.

In WAT, adipocyte precursor cells have been recently described to reside in the vasculature (Tang et al., 2008). Interestingly, a comprehensive *in situ* hybridization study of Fstl1 transcript expression in murine embryogenesis indicates particular enrichment of Fstl1 transcript in the vasculature (Adams et al., 2007). For example, in the developing gut Fstl1 expression is high in the sub-epithelial vascular presumptive lamina propria (Adams et al., 2007). In the developing lung, high levels of Fstl1 expression are localized to mesenchymal cells in the vasculature developing around alveoli (Adams et al., 2007). At mid-gestation stages of murine embryogenesis, Fstl1 is strongly expressed in the developing somites and in the mesenchymal component of other tissues but is largely excluded from epithelium (Adams et al., 2007). The possibility that Fstl1 is a marker of mesenchymal cells, particularly those with stem cell characteristics, is further supported by the observation that intestinal mesenchmymal stem cell lines and putative intestinal mesenchymal stem cells *in vivo* display dramatically elevated expression of Fstl1 compared to that of various other cell lines or surrounding cell types, respectively (Geske et al., 2008). Fstl1 transcript has also been reported to be expressed in C2C12 myoblasts and decreased during their myogenic conversion, albeit apparently not to the same all-or-nothing extent we observe for adipogenesis (Rosenberg et al., 2006). Together, these observations raise the notion that downregulation of Fstl1 transcript level may be a conserved event in the differentiation of mesenchymal stem-like type cell precursors to their respective terminally differentiated phenotypes. The adipose stromal compartment of adult tissue contains putative stem cell populations that can be induced to differentiate *in vitro* to osteogenic, adipogenic, myogenic, neuronal and chondrogenic lineage (Zuk et al., 2002). Precursor mesenchymal/stromal cell types (i.e. Swiss 3T3 and C3H10T1/2 cell lines) undergo differentiation to muscle, cartilage and fat cells after treatment with 5-aza-CR (Taylor and Jones, 1979). Of the agents tested we find that 5-aza-CR led to the most dramatic alteration of Fstl1 transcript level in 3T3-L1 preadipocytes, with a ~90% downregulation that was also reflected at the protein level. Demethylation is nearly exclusively reported to enhance transcription of cognate genes (Christman, 2002; Jones, 1985). We therefore surmise that the decreased level of Fstl1 transcript in 3T3-L1 preadipocytes by 5-aza-CR may be an indirect result of the demethylation-mediated activation of a currently unidentified regulatory gene. This gene would in turn repress gene transcription of select preadipocyte genes such as Fstl1. This may be one step in a regulatory cascade whereby 5-aza-CR treatment induces mesenchymal stem-like cells to differentiate along distinct lineages.

Studies on transcriptional mechanisms of gene activation in adipogenesis are frequently reported. In marked contrast, very few studies to date have attempted to assess mechanisms of selective gene expression in preadipocytes vs. adipocytes. A very recent report utilizing microarray analysis of KLF15-overexpressing 3T3-L1 adipocytes reported that, of ~6000 genes examined, 247 were downregulated in KLF15-expressing adipocytes compared to control adipocytes (Nagare et al., 2009). Combining this transcriptional profiling data with a that from a ChIP-on-Chip study resulted in the identification of two genes, adrenomedullin (Adm) and adenine phosphoribosyl transferase (Aprt), that were negatively regulated by direct promoter binding of KLF15 (Nagare et al., 2009). This observation and our data herein suggest that in adipocytes KLF15 may serve to actively suppress a subset of preadipocyte-expressed genes, and it also reinforces the notion that KLF15 has both activating and repressive roles in the adipocyte lineage. However, unlike the near all-or-nothing expression of Fstl1 transcript in 3T3-L1 preadipocytes vs. differentiated adipocytes, adrenomedullin transcript expression decreases by only approximately two-thirds during adipogenesis of 3T3-L1 preadipocytes to adipocytes (Nagare et al., 2009). In addition to the transcriptional effects of KLF15, additional genetic and epigenetic mechanisms may function to initiate downregulation of Fstl1 and other preadipocyte genes during adipogenesis and act to ensure their persistent repression in mature adipocytes.

While the role of Fstl1 in myogenesis is also not known, an interesting mechanism for decreased of Fstl1 transcript during myogenesis has been uncovered. The onset of myogenesis is accompanied by enhanced expression of MyoD, a well-established master regulator of muscle differentiation. Use of an *in vitro* estrogen-mediated activation of a MyoD-ER fusion protein to identify MyoD-regulated transcripts revealed negative regulation of Fstl1 transcript by MyoD (Bergstrom et al., 2002; Rosenberg et al., 2006). It was determined that MyoD enhances transcription of miR-206, one of several identified muscle-specific microRNAs. miR-206 in turn targets Fstl1 transcript for degradation *via* interaction at the Fstl1 3′ untranslated region (Rosenberg et al., 2006). As miR-206 is muscle specific, it would appear that this particular microRNA mechanism would not be applicable to downregulation of Fstl1 expresssion in adipogenesis. To date, several microRNAs have been investigated in detail in regard to adipogenesis, most notably miR-103, miR-143 and miR-let-7 (Sun et al., 2009; Takanabe et al., 2008; Xie et al., 2009). Analysis with TargetScan software fails to identify binding sites for these within the Fstl1 transcript. However, it is possible that other microRNAs expressed and/or regulated in the adipocyte lineage could be involved. Curiously, the 3′ untranslated region of the human Fstl1 transcript itself encodes a microRNA, mir-198, one of the few microRNAs present in a protein-coding transcript. However mir-198 does not appear conserved in murine (Lewis et al., 2005). Production of mir-198 may lead to degradation of the Fstl1 transcript, although this has not been formally examined. The only functional role for mir-198 to date is restriction of HIV-1 replication in monocytes by a mechanism that apparently involves repression of cyclin T1 (Sung and Rice, 2009). The function of mir-198 within the adipocyte lineage has not been examined. Of interest, however, in a study of expression level of 155 different micro RNAs in human omental and subcutaneous adipose tissue, miR-198 was unique in that its level of expression showed the highest degree of correlation with mean omental adipocyte diameter. An inverse correlation was noted wherein smaller omental adipocytes expressed higher levels of mir-198, and vice versa (Kloting et al., 2009).

BMP4 is one important signaling pathway involved in the differentiation of mesenchymal lineages (Bowers et al., 2006;

Bowers and Lane, 2007; Butterwith et al., 1996; Otto et al., 2007; Tang et al., 2004). For example, treatment of multipotential murine CH310T1/2 mesenchymal cells with BMP4 primes these cells for greatly enhanced adipocyte conversion upon subsequent exposure to adipogenic inducing agents (Butterwith et al., 1996; Tang et al., 2004). We did not, however, observe regulation of Fstl1 transcript by BMP4 treatment of 3T3-L1 preadipocytes. Nonetheless, a possible link between Fstl1 and BMP4 signaling in regard to adipogenesis remains to be more fully explored. This is particularly so in light of reports in zebrafish and chick development indicating that Fstl1 may impact BMP4 signaling pathways (Amthor et al., 1996; Esterberg et al., 2008; Towers et al., 1999). In zebrafish, BMP4 signaling acts in early development to establish mesodermal cell identity. Transcripts for Fstl1 orthologs are expressed in zebrafish axial and paraxial mesoderm during late gastrulation and segmentation stages (Esterberg et al., 2008). In this model, morpholino-based knockdown of Fstl1 orthologs enlarged the tailbud domain and led to increased expression of markers indicative of enhanced BMP4 signaling (Esterberg et al., 2008). Follistatin can act by directly binding to and inhibiting function of the TGF-β superfamily member activin (Chen et al., 2006). To our knowledge, whether Fstl1 binds activin or functions by an analogous mechanism has not been reported nor explored. This may be unlikely given the low degree of sequence conservation of the follistatin-like module of Fstl1 with that of follistatin, and the fact that follistatin has three such modules in tandem vs. the single follistatin-like domain of Fstl1. Additional studies indicate that the expression of Fstl1 in embryogenesis may also involve interplay of the hedgehog signaling pathway (Amthor et al., 1996; Towers et al., 1999), which has a described regulatory role in adipogenesis (Cousin et al., 2007). It remains to be determined if and where in development and differentiation of adipocytes Fstl1 fits in regard to the complex cell signaling networks mediated by BMP4 and sonic hedgehog.

As cells transition from preadipocytes to terminally differentiated adipocytes, a major phenotypic alteration is cessation of cell proliferation due to cell cycle withdrawal (Gregoire, 2001; Gregoire et al., 1998). This suggests a positive correlation of Fstl1 expression with cell growth/proliferation. However a number of studies, particularly in cancer cells, have reported that Fstl1 is anti-proliferative and suggested a tumor suppressor role for Fstl1. Fstl1 transcript level was observed to be decreased in cancer vs. normal cell lines and to decrease following experimental transformation of cells with select oncogenes (Mashimo et al., 1997; Shibanuma et al., 1993; Sumitomo et al., 2000). Ectopic expression of Fstl1 in cancer cells or transformed fibroblasts reduced proliferation and/or invasiveness (Johnston et al., 2000; Sumitomo et al., 2000). A number of other studies support an anti-proliferative role for Fstl1. This includes inhibited proliferation of human lung cancer cells (Sumitomo et al., 2000), vascular smooth muscle cells (Liu et al., 2006), a rheumatoid synovial cell line (E11), Ovca420 ovarian (Chan et al., 2009), and AN3CA endometrial cancer cells (Chan et al., 2009). In the latter instance, ectopic expression of Fstl1 enhanced apoptosis, and reduced cell migration and invasion (Chan et al., 2009). These observations on the anti-proliferative effects of Fstl1 are consistent with aforementioned studies of zebrafish morphants that reported enhanced proliferation and tissue expansion upon knockdown of Fstl1 orthologs (Esterberg et al., 2008). Overall, these data suggest that Fstl1 might function during embryogenesis and in cancer cells to inhibit cell growth and/or migration. In contrast to the anti-proliferative role described for Fstl1 in cultured cells, studies reported to date in human clinical cancer samples do not reveal a consistent pattern of Fstl1 deregulation indicative of tumor suppressor function. For example, Fstl1 transcript is downregulated in primary clear-cell renal-cell carcinoma (ccRCC) compared with adjacent normal kidney tissue (Tan et al., 2008). It is also decreased in metatstatic ccRCC vs. primary ccRCC tumors (Tan et al., 2008) and in majority of ovarian and endometrial cancers (Chan et al., 2009). On the other hand, Fstl1 is reported upregulated in primary and secondary glioblastoma multiforma with normal glial cells negative for Fstl1 expression (Reddy et al., 2008). Analysis of prostate cancers revealed that overexpression of Fstl1 was associated with higher metastatic potential (Nagare et al., 2009). Our observations of high expression of Fstl1 in preadipocytes and its marked decrease in early adipogenesis, a time coincident with their cell cycle withdrawal, and our observed transient increase in Fstl1 transcript coincident with the clonal expansion period of adipogenesis, appears consistent with a pro-proliferative role for Fstl1 in the adipocyte lineage.

Fstl1 has an emerging role as a signaling molecule in immunity. As such, preadipocyte expression of Fstl1 within WAT may contribute to the complex interplay between WAT and the immune system. Reports to date on the role of Fstl1 in immune response, however, appear contradictory in nature in that both anti- and - pro- inflammatory functions have been described. In respect to the former case, Kawabata and colleagues reported that treatment with recombinant Fstl1 ameliorates joint inflammation in a mouse model of arthritis leading to decreased expression of disease–promoting genes such as c-*fos* and IL-6 (Kawabata et al., 2004). A second study on rheumatoid arthritis reported recombinant Fstl1 expression downregulates synovial production of MMP-1, MMP-3 and prostaglandin E2 (Tanaka et al., 2003). Fstl1 is over-expressed during both the induction and maintenance phase of heart allograft tolerance with ectopic Fstl1 expression in this model reducing production of several pro-inflammatory cytokines and prolonging allograft survival (Le Luduec et al., 2008). On the other hand, various data support a pro-inflammatory function for Fstl1. In apparent contrast to the report of Kawataba, Miyamae and colleagues reported Fstl1 to be increased in a collagen-induced murine model of arthritis (Miyamae et al., 2006). They found that Fstl1 exacerbates arthritis when delivered by gene transfer, apparently by enhancing T-cell IFNγ production, and that neutralization of Fstl1 inhibited arthritis and diminishes IFNγ and CXCL10 levels in arthritic joints (Miyamae et al., 2006). Fstl1 transfection into macrophages and fibroblasts increased levels of IL-1β, TNFα, and IL-6 (Miyamae et al., 2006). It has also been reported that Fstl1 and/or its autoantibodies are over-expressed in synovial tissues from rheumatoid arthritis patients (Ehara et al., 2004; Tanaka et al., 1998). Circulating Fstl1 has recently been proposed as an inflammatory serum biomarker in humans and increased serum levels reported in acute coronary syndrome (Widera et al., 2009). It remains to be determined

whether expression of Fstl1 by preadipocytes plays a paracrine signaling role in the regulation of cytokine production by adipocytes or macrophages within WAT.

Given the fact that myocytes and preadipocytes have the same mesodermal origin, recent reports of Fstl1 function and mechanism(s) in muscle may have implications for the role of Fstl1 in the adipocyte lineage. Fstl1 transcript was found to be upregulated in hearts of transgenic mice with cardiac-specific ectopic Akt expression. These observations led to the discovery that Fstl1 is secreted by neonatal rat ventricular cardiomyocytes (Oshima et al., 2008). Ectopically expressed Fstl1 protects these cells from hypoxia-induced apoptosis and protects myocardial tissue from effects of ischemic stress (Oshima et al., 2008). Ectopic expression of Fstl1 in cardiomyocytes *in vitro* increased levels of phosphorylated Akt and enhanced ERK phosphorylation, two key protective signals for myocardium (Oshima et al., 2008). In gastrocnemius muscle Fstl1 is upregulated by ischemia to promote revascularization (Ouchi et al., 2008). *In vitro* studies of human umbilical vein endothelium cells revealed Fstl1 facilitates endothelial network formation, promotes cell migration and suppresses apoptosis (Ouchi et al., 2008). The initial stage in formation of the "primitive organs", which are described to precede the appearance of *bona fide* adipose tissue during development, is formation and expansion of dense capillary networks. Enhancement of blood supply *via* vascularization within WAT is also a key step that is required for the expansion of adipose mass in obesity and to prevent hypoxia therein (Rupnick et al., 2002). It is possible that Fstl1 functions within WAT to promote or maintain vascularization and/or as a preadipocyte-secreted cell survival factor.

In conclusion, reports of the last few years have illuminated Fstl1 expression and action in muscle, immune response, embryonic development and cancer, indicating a complex role(s) for Fstl1 therein. We postulate that preadipocyte-expressed Fst1l may exert similar pleiotropic actions in adipogenesis and adipose tissue function.

4. Experimental procedures

4.1. *Cell culture and differentiation*

3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM with 10% calf serum (CS). For differentiation, cells were treated at 2 days post-confluence with DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (Mix), and 1 μM dexamethasone (Dex) for 48 h. After induction, cultures were maintained in 10% FBS medium. ScAP-23 cells were maintained in DMEM containing 10% CS and passaged prior to reaching confluence. For adipocyte differentiation, preconfluent ScAP-23 preadipocytes were cultured in DMEM containing 10% FBS in the presence of the adipogenic inducers 0.5 mM Mix, 1 μM Dex, 17 nM insulin, and 0.2 mM indomethacin for 70 h. Agents were then removed and cultures maintained in DMEM containing 10% FBS and 17 nM insulin for an additional 4 days. The AD2.7AC cell line (Lecka-Czernik et al., 1999) was obtained from Dr. B. Lecka-Czernik (University of Toledo College of Medicine, Toledo, OH). Their culture and adipogenic induction is as for ScAP-23. For culture and differentiation of primary white adipocytes, WAT collected from male C57BL/6 mice or Sprague–Dawley rats was digested with 1 mg/ml of type I collagenase for 40 min at 37 °C with shaking. Following digestion, material was filtered through a 300 μm pore size nylon mesh (Sefar America Inc., Depew, NY) and filtrate centrifuged at 2000 rpm for 5 min. The floating adipocyte fraction was removed and the pellet of preadipocyte-containing stromal-vascular fraction (SVF) cells was resuspended in DMEM containing 10% FBS and plated. Upon confluence cells were either harvested or subjected to culture in differentiation media that consisted of DMEM containing 10% FBS, 0.5 μM Dex, 0.25 mM Mix, and 17 nM insulin for 3 days. After this, media was removed and cell cultures were maintained in DMEM containing 10% FBS and 17 nM insulin for an additional 4 days. For differentiation of a brown preadipocyte cell line (termed herein WT-BAT) obtained from C.R. Kahn (Joslin Diabetes Foundation, Harvard Medical School, Boston, MA), cells were cultured to confluence in DMEM supplemented with 10% FBS, 20 nM insulin and 1 nM triiodothyronine (T3) (Klein et al., 2002). Confluent cells were incubated in differentiation medium that contained 0.5 mM Mix, 0.5 μM Dex and 0.125 mM indomethacin for 2 days. After induction, cells were maintained in medium supplemented with 20 nM insulin and 1 nM T3 for an additional 4 days. The culture and adipogenic induction of mBAP-9 cells is as for WT-BAT. A manuscript more fully describing derivation and characterization of the mBAP-9 cell line is in preparation. COS were cultured in DMEM supplemented with 10% FBS.

For treatment with TNFα, 3T3-L1 adipocytes were incubated with 10 ng/ml TNFα for the indicated times. For assessment of Fstl1 regulation by components of the adipogenic cocktail, post-confluent 3T3-L1 preadipocytes were treated with 1 μM Dex or 0.5 mM Mix or the combination of Mix and Dex for 48 h. For assessment of regulation of Fstl1 transcript by a panel of various other agents, confluent 3T3-L1 preadipocytes were maintained in fresh DMEM with 10% CS for 24 h as control or treated for 24 h with either 10 nM retinoic acid; 2 ng/ml basic fibroblast growth factor (b-FGF); 10 ng/ml interleukin-6 (IL-6); 100 ng/ml bone morphogenetic protein 4 (BMP4); 200 μM indomethacin; 10 μM forskolin; 0.5 mM Mix; 10 nM sodium butyrate; 1 mM 5-azacytidine (5-aza-CR), 10 nM T3 (triiodothyronine); 100 ng/ml VEGF (vascular endothelial cell growth factor); 1 μM dexamethasone; 1 nM dibutyryl cAMP (cyclic adenosine monophosphate); 5 μM tamoxifen; 100 ng/ml LPS (lipopolysaccharide); 10 ng/ml TNFα; 1 μM TSA (trichostatin A); 5 μg/ml PPARγ ligand 15-deoxy-Δ12, 14-prostaglandin J2. For 5-aza-CR analysis, 3T3-L1 preadipocytes were treated with 1 mM 5-aza-CR for the indicated times.

4.2. *RNA preparation, Northern blot analysis, DNA array hybridization and Q-PCR*

Total RNA was extracted with TriZol Reagent (Invitrogen Corp) according to manufacturer instruction. For Fstl1 expression in murine tissues, 8-week-old C57BL/6 male mice were utilized, with all animal treatments conducted with approval of the University of Toledo College of Medicine Institutional Animal Care and Use Committee. Fractionation of whole adipose tissue was performed by digestion with 1 mg/ml of type I

collagenase for 40 min with shaking at 37 °C. Following digestion, material was filtered through a 300 μm pore size nylon mesh (Sefar America Inc., Depew, NY) and filtrate centrifuged at 2000 rpm for 5 min. Floating adipocyte fraction was collected as adipocyte fraction (AF) and the pellet was collected as SVF. For array analysis, duplicate mouse ResGen GeneFilter Arrays (Invitrogen Corp.) containing 5184 genes, were processed according to manufacturer's instructions. Filters were hybridized with [α-^{33}P] dATP labeled cDNA probes synthesized from 8 μg of total RNA from 3T3-L1 preadipocytes and adipocytes. For Northern blot analyses, 5 μg of total RNA was electrophoresed in 1% agarose formaldehyde gels in MOPS buffer and transferred to Hybond-N nylon membrane (GE Healthcare, Piscataway, NJ). Hybridizations were performed under high stringency conditions in ExpressHyb (BD Biosciences Clontech), according to the manufacturer's directions. Probes were labeled with [α-^{32}P] dATP using a Megaprime DNA labeling system (Amersham Biosciences). After washing, membranes were exposed at −80 °C to Kodak Biomax film with a Kodak Biomax intensifying screen.

For Q-PCR analysis, total RNA was subject to purification with an RNeasy RNA purification kit with DNase I treatment (Qiagen Corp., Valencia, CA) and 4 μg used for first strand cDNA synthesis with SuperScript II RNase H (−) reverse transcriptase (Invitrogen Corp.) and an oligo(dT)-22 primer. Q-PCR was conducted with an ABI 7500 Q-PCR System. Target cDNA levels were analyzed by SYBR green-based Q-PCR in 25 μl reactions containing 1× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM each forward and reverse primers, and 10 ng of cDNA. Q-PCR primers were designed to span introns and sequences are available on request. Transcript expression was normalized against Gapdh transcript level except when otherwise stated. In all cases the same amount of input RNA/cDNA was used in side-by-side comparisons. The cycle threshold value was generated using ABI PRISM 7500 SDS software version 1.2 and exported to an Excel spreadsheet to calculate fold differences. Statistical analyses were conducted using single factor ANOVA.

4.3. Expression construct for HA-tagged Fstl1, transfection and Western blot analysis

An Fstl1 CDNA clone was obtained from ATCC (I.M.A.G.E. ID: 2647002). PCR with the following primers (5′ GCC AAG CTT CCG GAC CCG AGC ACG ATG TGG3′) and (5′ GGC GTC GAC TTA AAG AGC GTA ATC TGG AAC ATC GTA TGG GTA GAT CTC TTT GGT GTT CAC CTT3′) was used to generate the coding region of murine Fstl1 with a C-terminal HA tag. Primers introduced restriction sites (5′ HindIII and 3′ SalI, underlined) and HindIII/SalI restricted PCR product was purified and inserted into HindIII and SalI sites of pcDNA3.1 to produce the expression construct, Fstl1-HA-pcDNA.

For transfection, COS cells were seeded at 6×10^5 cells/100 mm dish. Ten micrograms of Fstl1-HA-pcDNA or pcDNA empty vector was transfected using the DEAE-dextran method. For Western blot analysis culture media and cell lysates were collected at 48 h post-transfection. Media was centrifuged at 5000 rpm for 3 min and cells harvested by lysis in TNN (+) buffer (10 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, supplemented with a protease inhibitor cocktail). Lysates were incubated on ice for 30 min with intermittent vortexing, supernatant collected *via* centrifugation, and protein content determined (Bio-Rad Laboratories). To examine Fstl1 protein expression in medium during 3T3-L1 cell differentiation, culture medium was harvested daily from the same well of cells in a 6-well plate, with medium changed 24 h pre-harvest. For assessment of TNFα regulation of Fstl1 in 3T3-L1 adipocytes, cells were incubated with or without 10 ng/ml TNFα for indicated times. To determine Fstl1 protein half-life, 3T3-L1 preadipocytes were treated with 5 μg/ml of cycloheximide and harvested at indicated time points. For Fstl1 protein regulation by 5-aza-CR treatment, 3T3-L1 preadipocytes were treated with or without 1 mM 5-aza-CR for 24 h and culture media harvested. For Western blot, proteins were separated in a 10% SDS–PAGE gels under reducing conditions and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore Corp.). Membranes were blocked 1 h in 5% nonfat milk/0.5% Tween 20 in PBS and then incubated with primary antibody for 1 h, followed by three 10 min washes. Anti-mouse Fstl1 antibody was purchased from R&D Systems, PPIA and tubulin antibodies were from Santa Cruz Biotechnology. Secondary antibody was incubated for 30 min followed by three 10 min washes. All washes were in 0.5% Tween 20 in PBS. Signal was detected by ECL Plus enhanced chemiluminescence (GE Healthcare) and by exposure to X-ray film except for quantitative studies of protein half-life for which digital data was acquired using a Kodak Digital Image Station.

4.4. Luciferase constructs and reporter assay

To prepare luciferase reporter constructs containing regions of the mouse Fstl1 promoter, murine liver genomic DNA was subject to PCR with primers designed based on murine Fstl1 genomic flanking region sequence available through the Ensembl database (www.ensembl.org). For the −280/+60 promoter construct, PCR was conducted with a promoter proximal primer (5′ GGC CTC GAG TGG CGG CAG CGA GTT AGA GGC) that included an XhoI site (underlined) spanning through 60-base pair (bp) of the Fstl1 transcript in combination with a promoter-distal primer (5′ GGC ACG CGT TGA TTT CTG TGA TTT CCC CCG C) including an MluI site (underlined). For the −350/+60 promoter construct, PCR was conducted with the same promoter proximal primer as for the −280/+60 promoter construct and a promoter-distal primer (5′ GGC ACG CGT CGA GTT CCTTCT GTT ACC CAC) including a MluI site (underlined). For the −791/+60 promoter construct, PCR was conducted with a promoter proximal primer (5′ GGC AGA TCT TGG CGG CAG CGA GTT AGA GGC 3′) including a BglII site (underlined) and a promoter-distal primer (5′ GGC GGT ACC GAA AGA GAT TGG GGA TCC ACAC 3′) including a KpnI site (underlined). For the −3922/+22 promoter construct, PCR was conducted with a promoter proximal primer (5′ GGC ACG CGT TTA TCA CCA GGC TCC GAG GAA G 3′) including an MluI site (underlined), and a promoter-distal primer (5′ CAG CCG GTA CCA ATG CAC ACG CAT TGC CAC) including KpnI site (underlined). The resulting PCR products were ligated into the corresponding restriction sites of the pGL2-Basic vector (Promega Corp, Madison, WI). Promoter constructs were confirmed by complete sequencing.

To assess transcriptional response of the murine Fstl1 promoter region, Fstl1 promoter-luciferase constructs were transfected into 3T3-L1 preadipocytes, either singly or in combination with a KLF15 expression construct, using Lipofectamine 2000 (Invitrogen Corp). Empty vector pDNA3.1 was included as needed to maintain equal mass of DNA *per* transfection and all transfections included a pRLNull *Renilla* luciferase construct (Promega Corp.) as an internal control. *Firefly* and *Renilla* luciferase activity was determined at 48 h post-transfection using a Promega dual luciferase kit and a Turner Industries luminometer (Promega Corp.), with values corrected against *Renilla* luciferase signals. Statistical analyses were conducted using single factor ANOVA.

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