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Cellular transdifferentiation into brown adipose-like cells by adenoviral-directed expression or stable transfection of HB-EGF and ADAM 12S

Sean R TAYLOR, Jamie R KLEMENTS, Michael G MARKESBERY, Katie D JOHNSON and Paul A HARDING*

Cell, Molecular and Structural Biology Program, Biology Department, Miami University, Oxford, OH 45056, USA

(author for correspondence*; hardinpa@miamioh.edu*)*

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Abstract

Adenoviral vectors directing the expression of HB-EGF and ADAM 12S were used to better understand the gene expression profile of cellular reprogramming into BAT-like cells compared to HB-EGF and ADAM 12S transfection. Human epidermoid carcinoma cells (A431) were co-infected with HB-EGF and ADAM 12S adenoviruses, monitored for fluorescence and lipid accumulation for 1-3 weeks. Cells were stained with Oil Red O and total RNA was harvested and analyzed with a human adipogenic RT^2 Profiler Array. Additionally, the gene expression profile of RNA from A431 control cells and A431 cells stably transfected with HB-EGF and ADAM 12S were analyzed and compared to the gene expression patterns of adenoviral HB-EGF and ADAM 12S infected cells. Adenoviral HB-EGF ADAM 12S coinfected A431 RNA exhibited significant up-regulation of PGC-1α, Klf3, Klf4 and FGF-2 mRNA and downregulation of C/EBPα, LMNA, GLUT4 and SRC mRNA. RNA derived from HB-EGF and ADAM 12S co-transfected A431 cells exhibited similar gene expression profiles with the human adipogenic array. Adenovirus HB-EGF and ADAM 12S co-infected cells transdifferentiate human A431 cells into a BAT-like phenotype and recapitulate findings with HB-EGF and ADAM 12S transfected cells. The findings presented in this study identify genes important in BAT-like cellular reprogramming and may have therapeutic implications to obesity and type 2 diabetes.

Keywords: HB-EGF, ADAM 12S, brown adipose tissue, white adipose tissue

Özet

HB-EGF ve ADAM 12S'in adenovirüsle yönlendirilmiş ifadesi veya kalıcı transfeksiyonu yoluyla kahverengi adipoz benzeri hücrelere hücresel transdiferansiyasyon

HB-EGF ve ADAM 12S transfeksiyonuyla karşılaştırıldığında HB-EGF ve ADAM 12S'in anlatımını yönlendiren adenoviral vektörler, BAT benzeri hücrelere hücresel yeniden programlama gen anlatım profilinin daha iyi anlaşılmasında kullanılmaktadır. İnsan epidermoid karsinoma hücreleri (A431) HB-EGF ve ADAM 12S adenovirüsleri ile ko-transfekte edildi, 1-3 hafta boyunca floresans ve lipid birikimi için izlendi. Hücreler OilRed O ile boyandı ve total RNA elde edilerek insan adipojenik RT^2 Profil Dizini ile analiz edildi. Ek olarak, A431 kontrol hücreleri ve HB-EGF ve ADAM 12S ile kalıcı transfekte edilmiş A431 hücrelerinden RNA gen anlatım profili analiz edildi ve adenoviral HB-EGF ve ADAM 12S ile enfekte edilmiş gen anlatım paternleri ile karşılaştırıldı. Adenoviral HB-EGF ADAM 12S ile ko-enfekte A431 RNA PGC-1α, Klf3, Klf4 ve FGF-2 mRNA anlatımında belirgin bir artış ve C/EBPα, LMNA, GLUT4 ve SRC mRNA anlatımında azalma gösterdi. HB-EGF ve ADAM 12S ko-transfekte edilmiş A431 RNA insan adipojenik dizin ile benzer gen anlatım profili sergiledi. Adenovirüs HB-EGF ve ADAM 12S ko-enfekte hücreler, insan A431 hücrelerini BAT benzeri fenotipe farklılaştırır ve HB-EGF ve ADAM 12S transfekte hücrelerin bulgularını tekrarlar. Bu çalışmada sunulan bulgular BAT benzeri hücresel programlamada önemli genleri tanımlamakta ve obezite ve tip 2 diyabet için terapötik çıkarımlar sunmaktadır.

Anahtar kelimeler: HB-EGF, ADAM 12S, kahverengi adipoz doku, beyaz adipoz doku

Introduction

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a potent growth factor that binds and activates EGF receptors (EGFR) and is involved in a number of normal physiological processes including cellular proliferation of a number of cell types, including hepatocytes, as well as pathological disorders that include hepatocellular carcinoma (*reviewed in Taylor et al., in press*). Furthermore, HB-EGF has been shown to be protective in necrotizing enterocolitis (NEC), a common gastrointestinal injury, likely utilizing the canonical EGFR pathway (Yu *et al.*, 2009).

Another disorder that HB-EGF has been implicated in is obesity (Taylor *et al., in press*). Obesity is characterized by accumulation of white adipose tissue (WAT) from food intake exceeding energy expenditure. Mammals possess at least two types of adipose tissue: WAT, which functions for energy storage and brown adipose tissue (BAT), responsible for heat generation. BAT mitochondria contain uncoupling protein 1 (UCP1) that increases energy expenditure by uncoupling oxidative metabolism from ATP synthesis.

Co-expression of heparin-binding EGF-like growth factor (HB-EGF) and a disintegrase and metalloprotease (ADAM) 12S stimulates lipid accumulation and expression of BAT genes (Zhou *et al.*, 2013). HB-EGF is processed by ADAMs (Higashiyama and Nanba, 2004) releasing soluble HB-EGF that binds EGF receptors (EGFR) while the HB-EGF transmembrane and intracellular domain (HB-EGF-C) migrates to the nucleus relieving a transcriptional repressor (Higashiyama and Nanba, 2004). ADAM12 deficient mice exhibit reduced interscapular BAT (Kurisaki *et al.*, 2003), while mice that overexpress ADAM12 exhibit increased adipose tissue in muscle (Kawaguchi *et al.*, 2002). An adenoviral directed approach has been taken to provide insight into the mechanism of HB-EGF and ADAM12S cellular transdifferentiation into BAT-like cells.

Materials and Methods

Cloning of recombinant adenoviral vectors

AdEasy XL Adenoviral vector system (Stratagene) was used directing expression of ADAM12S and HB-EGF. Human HB-EGF and ADAM12S cDNAs were cloned and sequenced as previously described (Zhou *et al.*, 2013). HB-EGF sense primer encoding an *EcoRV* site and an anti-sense primer encoding a *Xho1* site were designed to amplify HB-EGF cDNA (635bp), gel-purified and digested with *EcoRV* and *Xho1* and ligated with PShuttle-2 digested with EcoRV and XhoI and termed pShuttle-2-HB-EGF. ADAM12S sense primer encoding a *Spe1* site and anti-sense primer encoding a *Xho1* site were designed to amplify ADAM12S cDNA (2.1kbp) gel purified and digested with *Spe1* and *Xho1*and ligated with pShuttle1 digested with Spe1, Xho1 and termed pShuttle-1-ADAM12S. hHB-EGF and ADAM12S lacked a stop codon allowing incorporation of 3X FLAG and tag protein (HA), respectively. pShuttle-IRES-hrGFP-1 was used as a control vector (pShuttle-1-mock).

Transformation of BJ5183-AD-1 cells to produce recombinant Adenovirus plasmids

pShuttle-1- mock, pShuttle-2-HB-EGF and pSHuttle-1-ADAM12S were linearized by *PmeI* digestion and treated with alkaline phosphatase for 30 min (37ºC). 0.1µg of each linearized shuttle vector was added to BJ5183-AD-1 competent cells, and electroporated according to manufacturer, transferred to 1ml LB broth and incubated for 1hr (37ºC) while shaking (225 rpm). The transformation mix (50µl, 200µl, and 850µl) was plated on LB-Kanamycin (50µg/ml) plates and incubated overnight (37ºC). Small colonies (recombinants) were selected and analyzed by *PacI* digestion resulting in ~30kb and 3kb DNA fragments, indicating recombination occurred between the left arms resulting in Ad-mock, Ad-HB-EGF, and Ad-ADAM12S plasmids.

Preparation of adenoviral stocks of Ad-HB-EGF and Ad-ADAM 12S

Recombinant Ad-mock, Ad-HB-EGF and Ad-ADAM12S plasmids were transformed and propagated in XL10-Gold Ultracompetent cells. Recombinant Ad-HB-EGF and Ad-ADAM12S plasmids were digested with *PacI* and transfected into AD-293 cells (8 x 10^5 cells/60mm dish) using Lipofectamine™ 2000 (Invitrogen) as described (Zhou *et al.*, 2013). Cells were examined by fluorescent microscopy 48 hrs. post-transfection and incubated for 7 days (37ºC, 4% CO_2, humidified chamber). Growth media was removed from Ad-HB-EGF and Ad-ADAM12S transfected cells and stored (-80ºC). 0.5ml PBS was added to cells, scraped, subjected to four rounds freeze/thaw in dry ice-methanol bath and 37ºC water bath. Cells were centrifuged (12k x g 10 min at 4ºC) and supernatant collected and stored at -80ºC,

representing the primary viral stocks. Viral titer (pfu/ml) was determined by diluting Ad-HB-EGF and Ad-ADAM12S primary viral stocks (10^{-5} to 10^{-9}) in 1ml and added to $5x10^5$ cells/well of a six-well plate including a no virus control, incubated for 2 hours (37ºC), and overlaid with 5% SeaPlaque agarose and incubated for 14 days. Viral titers ranged between $6.9x10^7$–$1.3x10^8$ pfu/ml. Individual plaques were isolated and used for an additional round of purification in order to generate high titer virus (>10^{12} pfu/ml). Human epidermoid carcinoma (A431) cells (10^5 cells/60mm dish) were infected with 1ml of high titer (>10^{12} pfu/ml) Ad-HB-EGF or Ad-ADAM12S as well as co-infection with Ad-HB-EGF and Ad-ADAM12S and incubated for 1 – 3 weeks.

Western blotting

Cellular lysates from Ad-mock, Ad-HB-EGF, and Ad-ADAM12S infected cells (1 week post-infection) were analyzed as described (Zhou *et al.*, 2013).

Oil Red O staining

Oil Red O staining of mock, Ad-HB-EGF, and Ad-ADAM12S infected cells was performed as described (Zhou *et al.*, 2013).

Quantitative real-time RT-PCR array analysis

The RT^2 Profiler PCR Array System human adipogenesis PCR array (SABiosciences, Frederick, MD) was used to analyze gene expression using RNA from mock infected A431 cells and Ad-HB-EGF/Ad-ADAM12S co-infected cells. Three weeks post-infection, total cellular RNA was isolated using Trizol Reagent (Invitrogen) and RNeasy Mini Kit (SABiosciences) followed by RT^2 First Strand Kit (SABiosciences) using 3.8μg RNA per sample, in triplicate. Analysis was based on the $\Delta\Delta C_t$ method with normalization to PPARδ, EGR2 and FASN.

Statistical analysis of data

P-values were obtained using SABiosciences online data analysis tool. Fold change uncertainty was calculated by the following = fold change * ln(2) * sqrt(σx2/(nx)+σy2/(ny)).

Results

Recombinant ADAM 12S and HB-EGF protein synthesis

Western blot analysis of cellular lysates from A431 cells infected with Ad-ADAM12S resulted in a 68kDa immunoreactive protein (Fig. 1A, lane 2) while no ADAM12S immunoreactive proteins were detected in Ad-mock or Ad-HB-EGF infected A431 cells (Fig. 1A, lanes 1,3). Ad-HB-EGF infected A431 cells resulted in a 21kDa immunoreactive protein (Fig. 1B, lane 3) while no HB-EGF immunoreactive proteins were detected in Ad-mock or Ad-ADAM12S infected cells (Fig. 1B, lanes 1 and 2). Western blotting of cellular extracts from AD-mock, AD-ADAM12S, and AD-HB-EGF infected cells were incubated with a mouse actin antibody resulting in equivalent amounts of 43kDa immunoreactive actin proteins (Fig 1C).

Effects of Ad-ADAM 12S and Ad-HB-EGF co-infection

A431 Ad-mock, Ad-ADAM12S, Ad-HB-EGF infected A431 cells and Ad-HB-EGF/Ad-ADAM12S co-infected cells were examined 24h post-infection and resulted in fluorescent cells, demonstrating infectivity (Fig. 1D, panels 2-5) while non-infected cells lacked fluorescence (Fig. 1D, panel 1). Ad-HB-EGF/Ad-ADAM12S co-infected A431 cells stained positive for Oil Red O (Fig. 1E, panel 5) while A431 cells, Ad-mock, Ad-ADAM12S, Ad-HB-EGF infected A431 cells lacked Oil Red O staining (Fig. 1E, panels 1-4).

Differential gene expression of adipogenesis-related genes

RNA from Ad-HB-EGF/Ad-ADAM12S co-infected A431 cells resulted in upregulation of genes involved in the BAT and transdifferentiation pathways including peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) {↑15.7X}**,** Krüppel-like factor 3 and 4 (Klf3,4) {↑3.7X and ↑2.8X}, respectively and fibroblast growth factor2 (FGF2), {↑4.2X} (Fig. 2A and C) while genes involved in WAT were downregulated and included CAAT-enhancer-binding protein (C/EBPα) {↓5.9X}, lamin A/C (LMNA) {↓4.1X}, glucose transporter type 4 (GLUT4) {↓2.4X}, and Schmidt-Ruppin A-2 viral oncogene homolog (SRC) {↓3.9X} (Fig. 2 B). RNA from A431 control and HB-EGF/ADAM12S co-transfected cells was used to validate the findings from the adipogenesis array and resulted in upregulation of PGC-1α {↑30X}, Klf3 {↑5.1X}, Klf4 {↑2.1X}, and FGF2 {↑4.1X} (Fig. 2 D,F) and downregulation of C/EBPα {↓5.1X} LMNA {↓2.9X}, GLUT4 {↓2.8X}, and SRC {↓2.4X} (Fig. 2E).

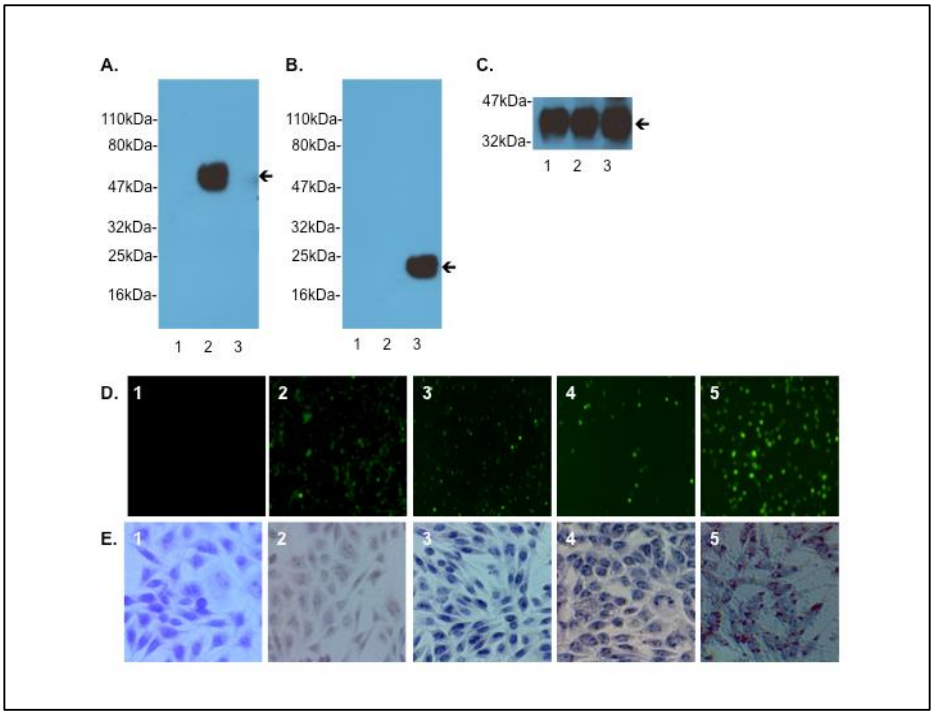

Figure 1. Characterization of Ad-HB-EGF and Ad-ADAM 12S infected A431 cells. Western blot analysis of cellular lysates from Ad-Mock, Ad-ADAM12S, Ad-HB-EGF infected A431 cells using an ADAM 12S antibody (panel A), HB-EGF antibody (panel B), and actin antibody (panel C). 24 hours post-infection, green fluorescent protein was monitored by fluorescent microscopy for non-infected A431, Ad-mock, Ad-ADAM 12S, Ad-HB-EGF, and Ad-ADAM 12S/Ad-HB-EGF infected A431 cells (panel D1 – D5, respectively). Oil Red O staining of non-infected A431, Ad-mock, Ad-ADAM 12S, Ad-HB-EGF, and Ad-ADAM 12S/Ad-HB-EGF infected A431 cells (panel E1 – E5, respectively). The data represent results reflective of three independent infections.

Effects of Ad-ADAM 12S and Ad-HB-EGF co-infection

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Table 1. Relative mRNA expression levels of HB-EGF/ADAM 12S infected or transfected A431 cells.

Gene	HB-EGF/ADAM 12S Adenovirus Infection (fold-expression)	HB-EGF/ADAM 12S Transfection (fold-expression)
KLF3	3.7	5.1
KLF4	2.8	2.1
FGF2	4.2	4.1
PGC-1α	15.7	30
C/EBP-α	-5.9	-5.1
GLUT4	-2.4	-2.8
LMNA	-4.1	-2.9
SRC	-3.9	-2.4

Numerical representation of genes differentially upregulated (KLF3, KLF4, FGF2, and PGC-1α) and downregulated (C/EBP-α, GLUT4, LMNA, SRC) by HB-EGF/ADAM 12S co-expression (n=3)/co-infection (n=2) with $p<0.05$.

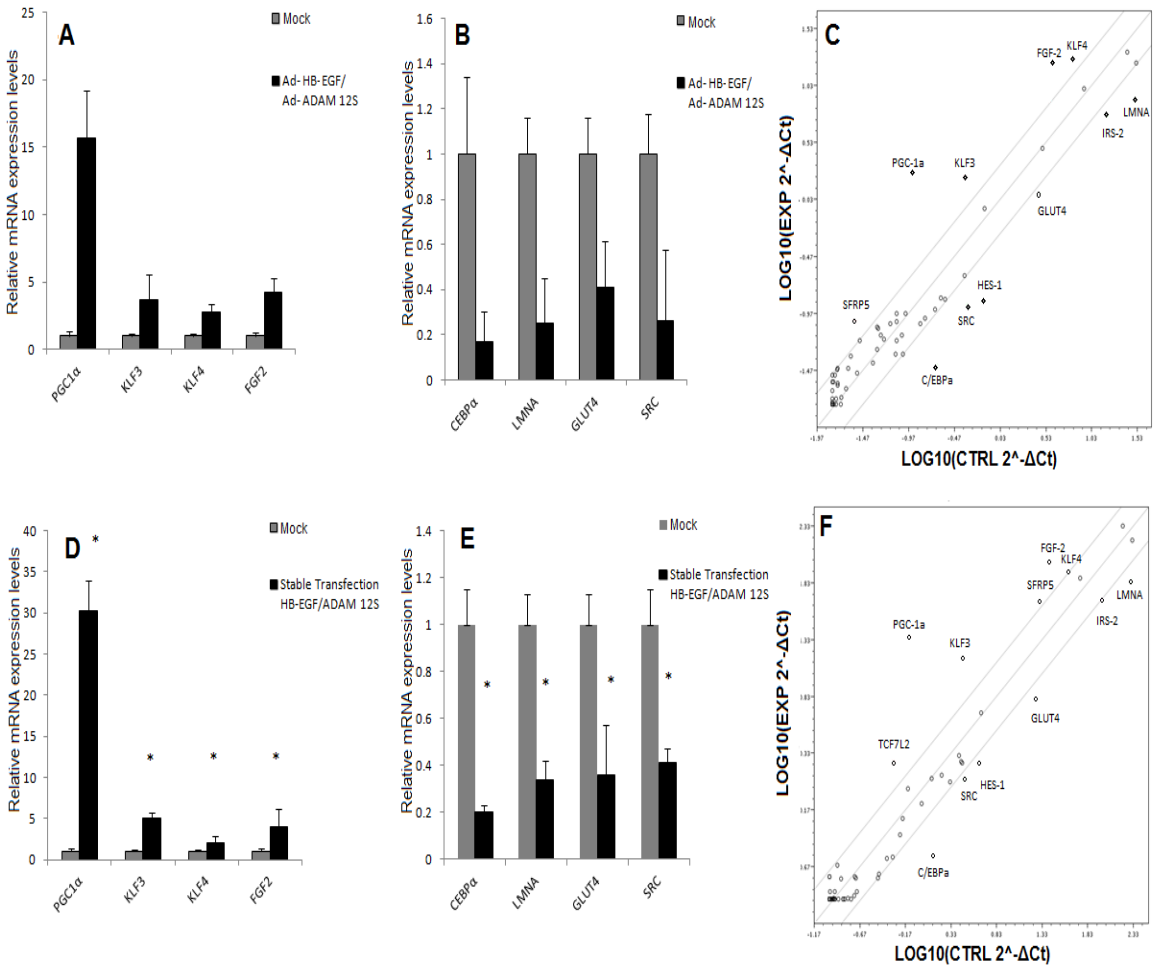

Figure 2. Differential gene expression of adipogenesis-related genes. RNA from Ad-mock (control) and Ad-HB-EGF/Ad-ADAM 12S co-infected cells (panels A-C) and A431 mock transfected and HB-EGF/ADAM12S stably co-transfected A431 cells (panels D-F) were analyzed using a human adipogenesis RT2 Profiler Array (n=2, n=3, respectively). Panels A and D represent upregulated genes from HB-EGF/ADAM 12S co-infected cells and HB-EGF/ADAM 12S stably transfected A431 cells, respectively, while panels B and E represent downregulated genes from HB-EGF/ADAM 12S co-infected cells and HB-EGF/ADAM 12S stably transfected A431 cells, respectively. Scatter plots of up- and downregulated genes from Ad-HB-EGF/Ad-ADAM 12S co-infected A431 cells compared to Ad-mock A431 cells (panel C) and HB-EGF/ADAM 12S stably transfected A431 cells compared to mock A431 cells (panel D).

Discussion

HB-EGF is a regulator of cell cycle progression (Nanba *et al.,* 2003; Toki *et al.* 2005). Recent findings demonstrated, HB-EGF/ADAM12S co-expressing cells exhibit lipid accumulation and BAT-like properties (Zhou *et al.,* 2013). To demonstrate these results are not an artifact of stable gene expression, A431 cells were co-infected with HB-EGF and ADAM12S adenoviruses.

A431 cells express two million EGF receptors (EGFRs) per cell and respond to HB-EGF resulting in increased cellular proliferation (Zhou *et al.* 2007). However, co-expression of HB-EGF/ADAM12S resulted in lipid accumulation rather than cellular proliferation, suggesting that the cells may have been reprogrammed. Several cell types have been reprogrammed into induced pluripotent stem cells by addition of FGF-2 (Durcova-Hills *et al.,* 2008). Klf4, an indicator of stemlike capacity, likely mediates FGF-2 cellular reprogramming of somatic cells to a pluripotent state (Durcova-Hills *et al.,* 2008). PGC-1α, a regulator of mitochondrial biogenesis, is highly expressed in BAT compared to WAT (Lowell and Spiegelman, 2000) and was significantly upregulated by HB-EGF/ADAM12S co-expression. Furthermore, Klf3 is upregulated by Klf4 (Patel *et al.,* 2006) and both were upregulated in HB-EGF/ADAM12S co-expressing cells. Klf3 represses C/EBPα (Sue *et al.,* 2008) that is essential for WAT but not BAT (Linhart *et al.,* 2001). PGC-1α and Klf3 are likely responsible for redirecting HB-EGF/ADAM12S co-transfected or co-infected cells toward a BAT-like phenotype.

HB-EGF/ADAM12S co-transfected or co-infected cells exhibited downregulation of C/EBPα, GLUT4, LMNA and SRC. C/EBPα, a transcription factor that modulates leptin expression (Hollenberg *et al.,* 1997), is required for WAT differentiation (Linhart *et al.,* 2001) suggesting that the reprogrammed A431 cells are not WAT. C/EBPα has been demonstrated to regulate GLUT4 (Kaestner *et al.,* 1990) and both C/EBPα and GLUT4 are downregulated by tumor necrosis factor alpha (TNF-α), an adipokine involved in the regulation of immune cells (Stephens and Pekala, 1991). This evidence suggests that C/EBPα and GLUT4 downregulation may be a result of HB-EGF and Klf3 expression. GLUT4 is expressed in WAT and BAT and present at lower levels in immature BAT (Dallner *et al.,* 2006). The HB-EGF/ADAM12S co-expressing cells in this study exhibited reduced levels of GLUT4 and a BAT-like phenotype. LMNA is important for maintaining the differentiated somatic state of cells in adulthood (Boguslavsky *et al.,* 2006), which may suggest that reduced LMNA expression is necessary for transdifferentiation in HB-EGF/ADAM12S cells. Overexpression of LMNA exhibit negative effects on adipocyte differentiation while decreased LMNA expression is associated with adipogenesis (Boguslavsky *et al.,* 2006). For example, LMNA deficient mice exhibit increased adiposity in muscle and bone (Tong *et al.,* 2011), supporting the hypothesis that reduced LMNA is necessary for transdifferentiation and adipogenesis. The tyrosine kinase (SRC) is involved in cellular proliferation and differentiation in A431 cells (Jin *et al.,* 1999) and inhibition of SRC in A431 cells reduced cellular proliferation (Schenone *et al.,* 2004). SRC is downregulated in HB-EGF/ADAM12S co-transfected/co-infected cells supporting previous findings indicating lack of cellular proliferation (Zhou *et al.,* 2013). In conclusion, these results suggest that adenoviral-directed HB-EGF/ADAM12S recapitulates the findings that HB-EGF and ADAM 12S co-transfected cells are capable of reprogramming A431 cells into BAT-like cells by activation of BAT genes and inhibition of WAT genes.

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