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Real-time monitoring of 3T3-L1 preadipocyte differentiation using a commercially available electric cell-substrate impedance sensor system

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ABSTRACT

Real-time analysis offers multiple benefits over traditional end point assays. Here, we present a method of monitoring the optimisation of the growth and differentiation of murine 3T3-L1 preadipocytes to adipocytes using the commercially available ACEA xCELLigence Real-Time Cell Analyser Single Plate (RTCA SP) system. Our findings indicate that the ACEA xCELLigence RTCA SP can reproducibly monitor the primary morphological changes in pre- and post-confluent 3T3-L1 fibroblasts induced to differentiate using insulin, dexamethasone, 3-isobutyl-1-methylxanthine and rosiglitazone; and may be a viable primary method of screening compounds for adipogenic factors.

1. Introduction

Label-free live cell monitoring provides an unhindered real-time view of whole cell biology. Real-time monitoring of adipogenesis using electric cell substrate impedance and capacitance based biosensor platforms have been reported for adipogenic human mesenchymal stem cells [1] and the mouse preadipocyte 3T3-L1 model [2], respectively.

In the capacitance based sensor developed by Lee et al. [2] cells were placed between two electrodes and the change in the dielectric constant (ε) was measured. This value is directly proportional to capacitance and is dependent on cell size, cell membrane potential and cellular content [3] making capacitance based sensors ideal to measure the differentiation of cells where gross cellular accumulation or novel biogenesis occurs; case in point, lipid accumulation during adipogenesis. However, lipid accumulation is but one property that can be measured to monitor adipogenesis. The morphology of cells dramatically changes during differentiation processes [4]. These changes in morphology can be measured in real-time using Electric Cell-Substrate Impedance Sensors (ECIS). These impedance based systems measure alternating current impedance differences between a smaller sensing and a larger counter current electrode; adherent cells remain viable and are cultured on the gold sensing electrode thereby passively blocking the current. The electrical impedance which results is registered by the sensor. Impedance is therefore affected by the shape, adhesion, or mobility of the adherent cells [5,6]. Both impedance based and capacitance based biosensors provide insights on what is happening to cells, and ideally an instrument that measures both these parameters would be advantageous.

The 3T3-L1 murine preadipocyte model is viewed as the gold standard to monitor unipotent cell differentiation to mature adipocytes, typically via supplementation with a cocktail of insulin, dexamethasone and 3-isobutyl-1-methylxanthine (INS/DEX/IBMX). The inclusion of the insulin sensitizer, rosiglitazone, has been reported to enhance differentiation [7]. Adipogenesis is typically gauged by end point monitoring by the formation of lipid droplets using Oil Red O staining. As 3T3-L1 differentiation has been shown to be sensitive to cell culture plastics and differentiation cocktail recipes [8], optimization of differentiation protocols through the use of end point assays can be laborious and inaccurate; real-time analysis offers defined benefits for the development of optimal, efficient differentiation protocols. This ultimately leads to more accurate results as a profile of cell growth, arrest or death [9] at any point during the differentiation process can be monitored.

When induced to differentiate, growth-arrested post confluent 3T3-L1 preadipocytes synchronously re-enter the cell cycle and undergo mitotic clonal expansion (MCE), mitotically dividing two to four times before differentiation. The DNA replication process

Abbreviations: INS, insulin; DEX, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; RTCA, Real-time Cell Analyser; ROSI, rosiglitazone.

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during growth is hypothesized to be required to make the promoter/enhancer elements available for the transcription of genes important for adipocyte differentiation [10].

The development of commercial real-time biosensors such as the ACEA xCELLigence Real-time Cell Analyser (RTCA) has revolutionised label-free cell biological analysis, giving more reliable medium to high throughput data regarding, amongst others, cell proliferation, migration [11], compound toxicity [9] and viral cytopathic effects [12]. Here we report the use of the ACEA xCELLigence RTCA Single Plate (SP) system to monitor the primary events of 3T3-L1 differentiation.

Based on published recommendations [7,8] we evaluated the ability of the ACEA xCELLigence RTCA SP to monitor growth and differentiation of 3T3-L1 preadipocytes in real-time in a 96 well assay format. The proprietary 96 well E-plates used in the experiment are designed with gold microelectrode arrays at the base of each well that measure electrical impedance at given time points (Z_i) in relation to a background reading (Z_0) to calculate arbitrary cell index (*CI*) units as:

$$CI = \frac{Z_i - Z_0}{15}$$

The xCELLigence monitoring software records the *CI* values which can be used to calculate theoretical doubling time (as cell density increases, so does *CI*) however as these values are affected by cell properties such as shape, adhesion, and mobility of cells, the system can be used to monitor morphological changes.

2. Methods

2.1. Reagents

Antibodies used in this study included: rabbit polyclonal anti-C/EBPβ IgG (sc-150), goat anti-rabbit IgG-horse radish peroxidase (HRP) (sc-2301) from Santa Cruz Biotechnology, Inc (USA), and AlexFluor® 488 goat anti-rabbit IgG (H + L) from Life Technologies (USA). Hoechst 33342 was provided by Invitrogen (USA), Trypsin/ethylenediaminetetraacetic acid (EDTA), propidium ioide, Dulbecco's Modified Eagles Medium (DMEM), L-glutamine, penicillin/streptomycin/amphotericin B (PSA), dexamethasone (DEX), 3-Isobutyl-1-methylxanthine (IBMX), Rosiglitazone (ROSI), Triton-X and Oil Red O were purchased from Sigma–Aldrich (USA). Fluorescence mounting medium was provided by Dako (USA). Foetal Calf Serum (FCS) was sourced from BioWest (France), insulin (INS) from Novo Nordisk (Denmark) and Clarity Western ECL Substrate from BioRad (USA). Unless stated otherwise, all reagents were of the highest grade and purity available.

2.2. Cell culture

Murine preadipocyte 3T3-L1 cells were routinely maintained in a basal media (DMEM supplemented with 5% (v/v) FCS, 1% (v/v) L-glutamine and 1% (v/v) PSA) in a humidified incubator at 37 °C, in a 5% CO_2 atmosphere and sub-cultured as necessary by routine trypsinization. Cell viability was determined by trypan blue staining. 3T3-L1 preadipocytes were freshly split 24 h prior to experimentation.

2.3. Differentiation of 3T3-L1 preadipocytes

Differentiation of 3T3-L1 cells was based on the protocol by Zebisch et al. [7]. Cells were grown to confluence (Day-2) and maintained in normal growth medium for a further 2 days. Two days post-confluence (Day 0), cells were washed with phosphate buffered saline (PBS, pH 7.4, 16 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl) and fed using adipocyte differentiation media. Differentiation media was composed of basal media supplemented with 10 μg/mL INS, 0.25 μM DEX, 0.5 mM IBMX and 2 μM ROSI. Two days post induction, media was aspirated and cells washed with PBS. Cells were supplemented with maintenance media containing 10 μg/mL INS for days 3–4 and then basal media.

2.4. Real time analysis using the xCELLigence system

The xCELLigence RTCA SP system was initialized, as per manufacturer's instructions, prior to commencement of the experiment by filling all 96 wells of the E-plate with the basal media (100 μL) and equilibrated at room temperature for 30 min. The plate was placed into the single plate (SP) station cradle (housed in a humidified incubator at 37 °C with a 5% CO_2 atmosphere) to establish a background reading. Cells were enumerated by trypan blue staining and plated at 1×10^4 cells per well in 50 μL aliquots in quadruplicate. Cells were allowed to settle for 30 min outside the incubator prior to returning the E-plate to the SP station. Growth was monitored by electrical impedance measurements using the RTCA SP as arbitrary *CI*. Cells were treated with either full differentiation media (as described above) or individual components of differentiation media (at the specified concentrations, as above) at 24, 48 or 60 h post-seeding. Standard differentiation media components, i.e. INS/DEX/IBMX/ROSI were added at 4x strength in 50 μl to account for dilution effects in predetermined wells. Duplicate clear plastic 96 well plates were prepared in the same manner to allow for comparative end-point Oil Red O staining and microscopy.

2.5. Oil Red O Staining

Following the xCELLigence experiment, plates were stained with Oil Red O as described by Ramirez-Zacarias et al. [13] with slight modifications. A working solution was prepared from a filtered 0.5% (w/v) stock solution with distilled water to a final concentration of 0.3% (w/v). Briefly, medium was discarded and cells were washed in PBS. Cells were then incubated for 5 min in 10% (v/v) formalin at room temperature after which fresh formalin was added and incubated at room temperature for a further hour. The plate was wrapped in parafilm and tinfoil to prevent drying. The formalin was removed and wells were washed with 60% (v/v) isopropanol. Wells were allowed to dry completely before the addition of Oil Red O working solution. The plate was incubated at room temperature for 10 min before removing the stain and washing repeatedly with sterile distilled water. Cells were viewed at 20× magnification (UOP BSZ500X Inverted microscope) under brightfield conditions, ScopePhoto 3.1 (ScopeTek) was used to capture images.

2.6. Cell cycle analysis

Pre-confluent and 2 days post confluent 3T3-L1 cells (as judged by light microscopy) were collected via trypsinization. Cells were stained with propidium iodide in the presence of RNAse as specified by Nunez [14]. After doublet discrimination gating to exclude cell aggregates, DNA content as a measure of PI fluorescence was detected by excitation with the blue laser at 488 nm and emission detected in the 610/20 channel flow cytometry using a Becton Dickson FACS Aria II flow cytometer. Cell cycle analysis was performed using FCSExpress 4 (De Novo Software) using built in cell cycle models.

2.7. Immunofluorescence staining

Cells were grown on borosilicate glass coverslips in 6 well culture plates (Nunclon™, Nunc). Following differentiation

treatments, cells were subjected to immunofluorescence staining. Medium was aspirated, and the cells were washed with PBS (3 × 10 min), before fixation for 30 min using a 4% (v/v) formaldehyde solution. Following fixation, cells were washed with PBS. Cells were then permeabilized for 5 min with 0.1% Triton X-100 (v/v) solution and washed with PBS (3 × 10 min). Coverslips were blocked for a minimum of 1 h at room temperature using a 1% (w/v) BSA/PBS solution. Coverslips were incubated with rabbit anti-C/EBPβ (1:500 dilution in 1% BSA/PBS) overnight at 4 °C. Thereafter the antibody solution was aspirated and coverslips washed with 1% BSA/PBS solution. Coverslips were incubated with AlexFluor® 488 goat anti-rabbit IgG (1:2000 dilution in 1% BSA/PBS) in the dark at room temperature for an hour before and finally washed with a 1% BSA/PBS solution (3 × 10 min). Hoechst 33342 solution (1:1000 in water) was used to stain nuclear material. The cover slips were dried before mounting with fluorescence mounting medium. Fluorophore labelled secondary antibody only staining was used as a control. Images were captured using the Zeiss AxioVert.A1 FL-LED Fluorescence Microscope and analysed using Zen Blue Microscope Software (Zeiss).

2.8. Western blot analysis

Cells were harvested and enumerated by trypan blue staining. Equal numbers of cells were lysed in Laemmli sample buffer supplemented with 5% (v/v) β-mercaptethanol (BioRad) at 95 °C for 10 min. Following SDS–PAGE electrophoresis [15] and transfer onto PVDF filter membranes (BioRad), membranes were blocked for 1 h at room temperature with 5% (w/v) skim milk in a TBS-Tween 20 (0.8% (w/v) NaCl, 0.24% (w/v) 1% (w/v) Tween-20) Tris, pH 7.6) buffer. Blocking was followed by an overnight incubation at 4 °C in rabbit anti-C/EBPβ (1:1000). The membrane was washed with TBS-T (3 × 15 min) and incubated with anti-rabbit-HRP (1:5000) for 1 h at room temperature. After washing, the membrane was developed with Clarity Western ECL Substrate according to the manufacturer's instructions. Images were captured using the UVTec UVIPro System.

Unless noted otherwise all experiments were performed in triplicate ($n = 3$).

3. Results and discussion

3.1. Confirmation of adipogenic initiation

CCAAT/enhanced binding protein β plays a significant role during adipogenesis [10,16]. C/EBPβ localization and levels were monitored by fluorescence microscopy and Western blot analysis to confirm adipogenic initiation upon stimulation with IBMX, dexamethasone, insulin and rosiglitazone (Fig. 1).

It is important to note that C/EBPβ has two major isoforms. It has been shown that isoform expression is differentially regulated at the translational level [17,18]. Isoforms have an expected relative mass of 38 and 18 kDa. Uninduced preadipocytes were shown to have minimal levels of the transcription factor, with a faint band of the larger isoform being observed in the uninduced lysate (Fig. 1A, Day 0 – Lane 1). Western blot analysis indicated that expression of C/EBPβ increased upon differentiation with maximum levels being observed in the first 24 h of differentiation, as observed Day 1 (Fig. 1A). These levels were shown to remain relatively constant for 4 days post induction (Fig. 1A, Days 2–5 – Lanes 3–6), and thereafter slowly started to diminish with minimal levels observed on Day 6 (Fig. 1A, Lane 7) of the sample set.

As expected, prior to the induction of differentiation the localization of C/EBPβ was cytoplasmic, with minimal nuclear staining on the Day 0 (Fig. 1B). The intensity of fluorescence signal in uninduced cells was particularly low. This low signal was expected as the levels of C/EBPβ in preadipocytes is expected to be minimal, as confirmed by Western blot analysis (Fig. 1A, Day 0). C/EBPβ localized to the nucleus (Fig. 1B, Day 1) as expected upon the induction of differentiation. This strong nuclear localization persisted for 48 h post-induction as highlighted in Days 1 and 2, Fig. 1B (red arrows). This nuclear staining was reduced 72 h post-induction. Punctate cytoplasmic structures (Fig. 1B, white arrow) were observed and the majority of cells had lost the nuclear C/EBPβ localization phenotype. Through days 4–6 post-induction the nuclear localization of C/EBPβ became less distinct (Fig. S1).

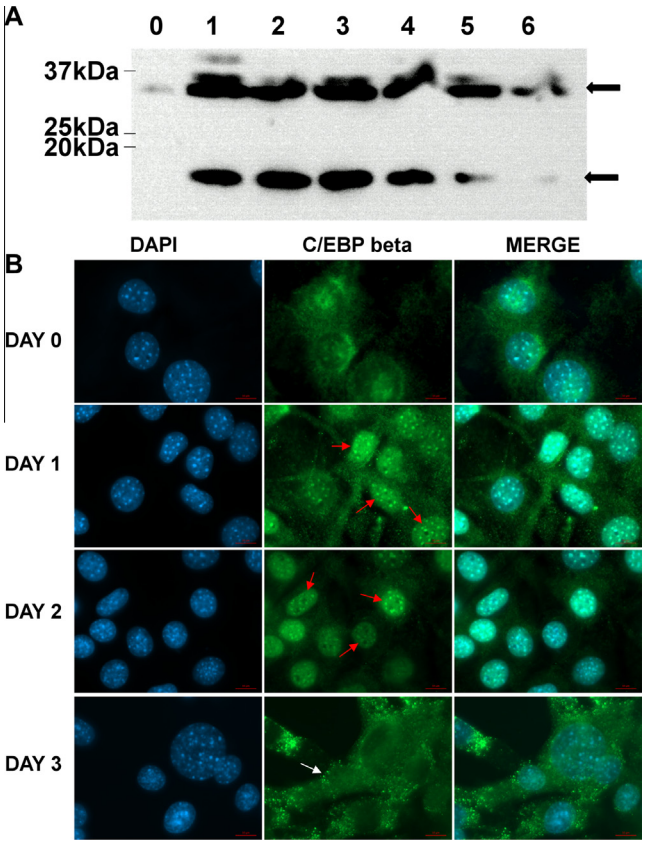

Fig. 1. Expression and localization of C/EBPβ during 3T3-L1 adipocyte differentiation. (A) Western blot analysis of C/EBPβ expression pre- (Day 0) and post- (Days 1–6) induction of adipocyte differentiation. (B) Immunofluorescence analysis of the localization of C/EBPβ through days 1–3 of 3T3-L1 adipocyte differentiation. Red arrows highlight nuclear localized of C/EBPβ during Days 1 and 2 post-induction, the white arrow highlights punctate structures observed on Day 3. Scale bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These results indicate that differentiation of the preadipocytes into adipocytes was occurring in the same manner as reported in literature. Tang and Lane [18] showed that the expression of C/EBPβ occurs rapidly after the induction of differentiation with maximum levels being reached 4 h after induction and maintained for a further 48 h. Levels of C/EBPβ then slowly start to decrease.

3.2. Real time monitoring of differentiation

The ability of the xCELLigence system to measure the adipogenic events following induction of differentiation was investigated. The requirement of cytostaticity (G_0) prior to induction of differentiation is well documented [19–21], and is typically achieved by growing cells to post-confluence. Despite the requirement of post-confluent cells for efficient differentiation it was hypothesized that the xCELLigence system would be able to

measure the morphological changes that accompany the induction of differentiation regardless of the general cell cycle state of the cells.

Cells were seeded at the same density in three separate experiments and induced to differentiate before reaching confluence, 24 h post-seeding (Fig. 2A), upon confluence, 48 h post-seeding (Fig. 2B) and once post-confluent, 60 h post seeding (Fig. 2C) as judged by the gradient of the *CI* curves and calculated doubling times. Analysis of cell cycle distribution by flow cytometry confirmed that pre-confluent cells were actively dividing, with a population found to be in the S phase of the cell cycle (~40%) while post-confluent cells had reached cytostaticity with the majority of the population (>90%) in $G_{0/1}$ as shown in the cell cycle analysis (Fig. 2D and E). Despite the reported requirement for cells to be cytostatic prior to induction of differentiation, the xCELLigence system was able to detect similar *CI* profiles for cells induced to differentiate regardless of their position in the cell cycle.

Mitotic clonal expansion, as it involves active cell growth, should be able to be monitored using the xCELLigence system. However, as shown in Fig. 2, the *CI* curves of cells induced to differentiate rapidly drop as opposed to the expected increase in *CI* values as a result of dividing cells. This dramatic drop was found to be reproducible and is thought to be a characteristic primary event in the *CI* curves of 3T3-L1 cells induced to differentiate. A similar *CI* curve of 3T3-L1 differentiation using the xCELLigence system was achieved by Melloni et al. [22]. The drop in the *CI* values upon induction of differentiation was speculated to be caused by a dramatic change in morphology. Cell death was ruled out as a cause for the drop in *CI* values as *CI* values did not decrease to 0 and cell viability was confirmed by microscopic analysis.

To confirm that these *CI* profiles were not artefacts of a single component of the differentiation cocktail and in fact as a result of the induction of differentiation, cells were individually treated with each of the components making up the differentiation cocktail. Each component plays a role in activation of the factors required for adipogenesis: Insulin acts through the insulin-like growth factor receptor (IGF-1). Activation of IGF-1 results in an increase of cyclic adenosine monophosphate (cAMP). Levels of cAMP are further increased through the activation of glucocorticoid pathways by glucocorticoids such as dexamethasone. The cAMP phosphodiesterase inhibitor, IBMX is used to further increase levels of cAMP. Cyclic AMP and IBMX decrease levels of Specificity protein 1 (Sp1), a transcription factor which represses the C/EBPα promoter [21]. To improve efficiency of differentiation, the insulin sensitizer and peroxisome proliferator-activated receptor γ (PPARγ) (another key adipogenic factor) agonist, rosiglitazone, has been included in the cocktail [7].

Cells treated with DMSO (0.3%), solvent controls (Fig. 3A), had minimal effect on the *CI* curve and growth of 3T3-L1 cells, while samples treated with full differentiation media resulted in the expected drop in the *CI* curve, as illustrated in Fig 3B. Both insulin (Fig. 3C) treatment and dexamethasone (Fig. 3D) treatment resulted in significantly altered *CI* profiles, while treatment with rosiglitazone (Fig. 3F) had no measurable effect, Treatment with IBMX (Fig. 3E) resulted in a *CI* profile similar to cells treated with the full differentiation cocktail. Interestingly, individual treatment of the cells with insulin, dexamethasone or IBMX resulted in an increased nuclear C/EBPβ localization (Fig. S2) however only IBMX treated cells resulted in a reduction of *CI* values comparable, but not identical, to the full differentiation *CI* profile as shown in Fig. 3G. This characteristic drop in *CI* values was unexpected, as upon induction of differentiation, cells are reported to re-enter the cell cycle and undergo active growth [10]. As the xCELLigence system is an impedance based sensor the dramatic reduction in *CI* values were hypothesized to be as a result of a morphological change in the cells. To illustrate that preadipocytes and differentiated adipocytes (Fig. 3H) are completely different cell phenotypes, in terms of cell type, cell size, cell shape and cell property, both preadipocytes and

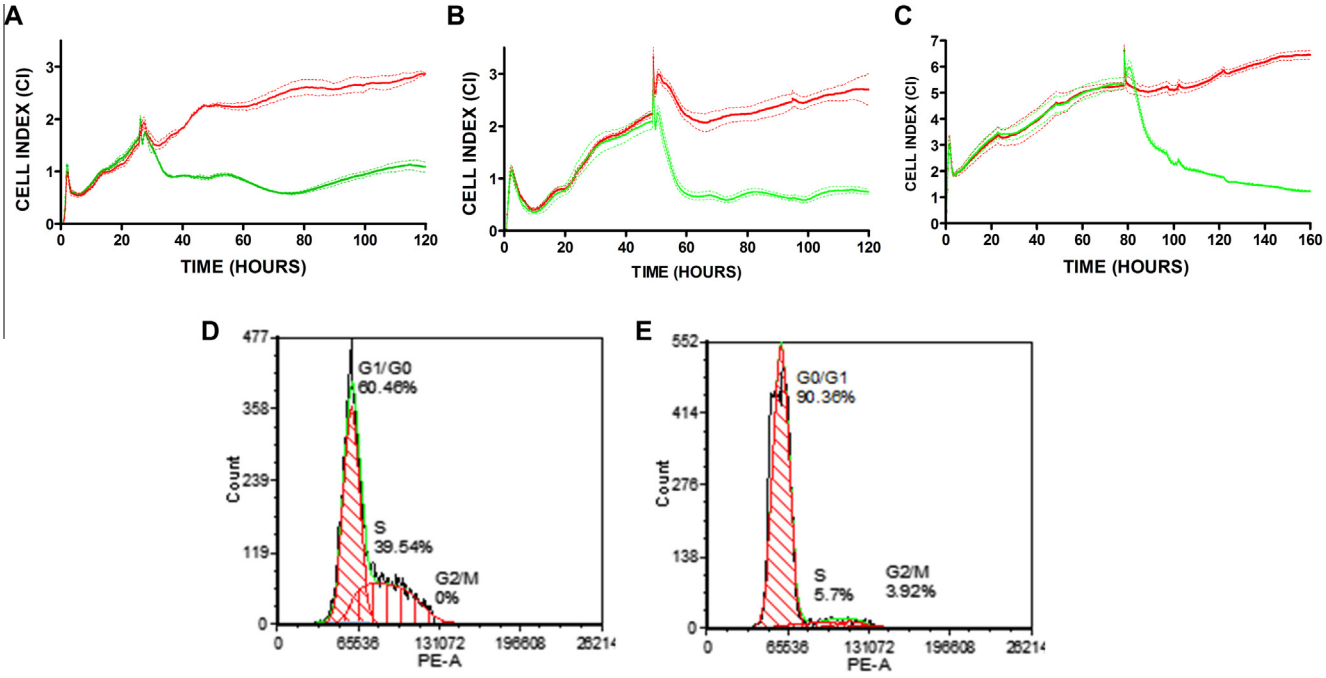

Fig. 2. The effect of confluency on the ability to detect differentiation events. (A–C): *CI* versus time curves of 3T3-L1 preadipocytes induced to differentiate at (A) pre-confluent (doubling time of 14.6 ± 0.58 h), (B) confluent (doubling time of 44.8 ± 0.6 h) and (C) post-confluent (doubling time of 283.6 ± 10.23 h) points as judged by the doubling times calculated 20 h pre-induction. Red curves indicate uninduced samples while green curves indicate samples induced to differentiate at points indicated by red vertical lines. Curves were plotted as an average of quadruplicate treatments. (D) Cell cycle analysis of pre-confluent and (E) post-confluent 3T3-L1 cells, as judged by light microscopy. Error bars show standard deviation ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

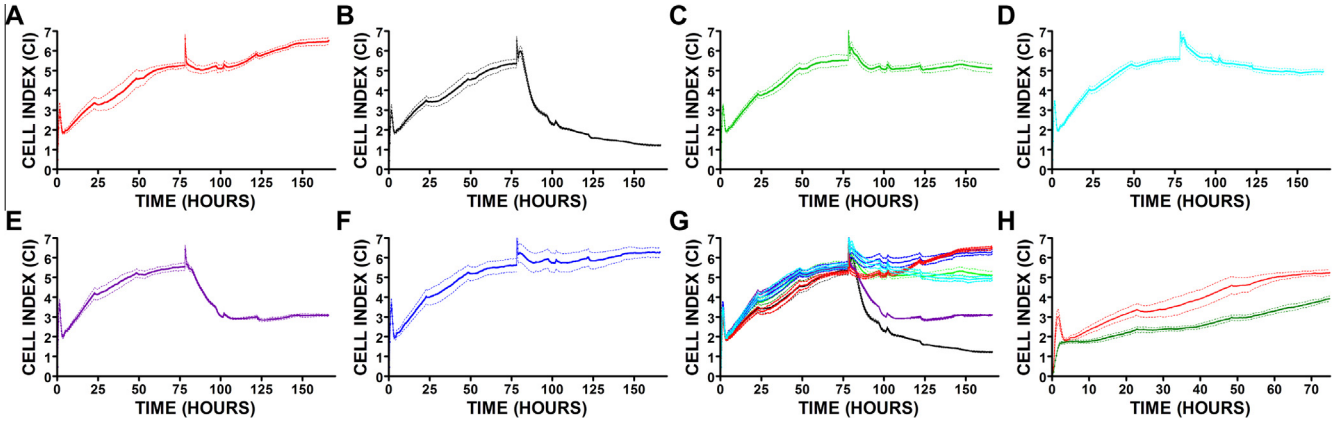

Fig. 3. Effects of individual components of differentiation cocktail on 3T3-L1 growth *CI* curves. Cells were treated at the same time with: (A) DMSO (control), (B) Differentiation cocktail (INS/DEX/IBMX/ROSI), (C) Insulin, (D) Dexamethasone, (E) IBMX, and (F) Rosiglitazone. (H) Preadipocytes (red) and differentiated adipocytes (green) were seeded at the same density to compare the resulting *CI* growth curves. All curves were plotted as an average of quadruplicate treatments. Error bars show standard deviation ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

14 days old differentiated adipocytes were plated at the same density onto the xCELLigence system and growth monitored and compared. As expected, the 2 cell types had different *CI* profiles as shown in Fig. 3H. Interestingly, adipocytes had lost the distinctive *CI* peak directly after plating the cells. This peak was routinely observed when plating preadipocytes on the system. Differentiated adipocytes had much lower *CI* values, and, as analysed using the xCELLigence software, grow much more slowly. Doubling time was calculated between 5 and 22 h post-seeding as illustrated in Fig. 3H. Adipocytes were found to have a doubling time of 37 ± 1.06 h while that of the preadipocyte progenitors were found to have a doubling time of 21.6 ± 3.28 h. Lower *CI* values further indicate that adipocytes are not as adherent as their fibroblast-like progenitors.

Fully differentiated 3T3-L1 cells (Fig. 4A) are typically rounded and have large lipid droplets, distinct from 3T3-L1 preadipocytes (Fig. 4B). However, microscopic analysis confirmed cells had adopted a spindle like morphology upon treatment with either full differentiation cocktail (Fig. 4C) or IBMX (Fig. 4F). This spindle shaped morphology was observed for approximately 48 h post induction, following which the cells become more characteristically rounded and accumulate lipid droplets. This morphological change seems to be a characteristic primary event in 3T3-L1 differentiation and may be a useful measurement for the screening of pro- or anti-adipogenic compounds. The individual treatment with insulin (Fig 4D) had minimal effect on the accumulation of lipid droplets, while treatment with dexamethasone (Fig. 4E), IBMX (Fig. 4F) or rosiglitazone (Fig. 4G) resulted in an increase in the accumulation of lipids. However, this was not measured by the xCELLigence system. This is expected as the system measures impedance and is therefore only able to measure changes as a result of cell size, morphology and membrane potential and not changes as a result of composition of cells.

We speculate, based on the *CI* curves resulting from the treatment of 3T3-L1 cells that IBMX is critical in allowing the induction of efficient differentiation and measurable associated primary morphological changes. As an aside, it has been reported that increased levels of cAMP lead to a spindle, fibroblast morphology in Chinese Hamster Ovary (CHO) cells [23]. We have utilized the ACEA xCELLigence RTCA SP system to successfully monitor the primary morphological changes that occur during 3T3-L1 adipocyte differentiation and successfully identified the instigating compound, IBMX, which causes this morphological change. In contrast, and in a sense in combination, to already established end point

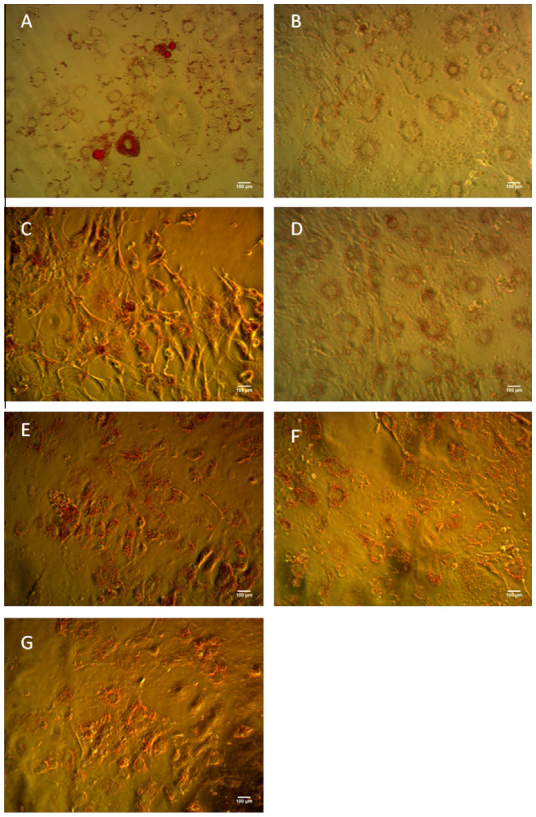

Fig. 4. Oil Red O staining of xCELLigence samples. (A) Terminally differentiated 3T3-L1 adipocytes. Preadipocytes treated with: (B) DMSO (control), (C) Differentiation cocktail, (D) Insulin, (E) Dexamethasone, (F) IBMX, or (G) Rosiglitazone Media. Scale bars = 100 μm, 200× magnification.

assays such as Oil Red O staining, immunofluorescence microscopy and Western blot analysis, real-time label-free monitoring provides a powerful non-invasive tool to streamline development of differentiation protocols.

By defining distinct *CI* profiles for cells with the potential to differentiate and undergo morphological shifts, impedance based real-time cell analysis can be used as an initial medium to high throughput screen for chemical morphogens which may have pro- or anti-differentiation activities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.123.

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