


# Human adipocyte differentiation and characterization in a perfusion-based cell culture device

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**Abstract** Adipocytes have gained significant attention recently, because they are not only functioning as energy storage but also as endocrine cells. Adipocytes secrete various signaling molecules, including adiponectin, MCP-1, and IL-6, termed collectively as “adipokines”. Adipokines regulate glucose metabolism, thereby play an important role in obesity, diabetes type 2, and other metabolic disorders. Conventionally, to study the secretory function, adipocytes are cultured *in vitro* in static conditions. However, static culturing condition falls short of mimicking the interstitial fluid flows in living systems. Here, we developed a perfusion device which allows dynamic culture of adipocytes under constant and mild flow using a double-layered fluidic structure. Adipocytes were cultured in the bottom layer while the culture media were constantly flown in the upper layer and perfused through a porous membrane that separates the two chambers. The porous membrane between the two chambers physically separates the cells from the flow stream while maintain a fluidic connection by diffusion. This setting not only provides continuous nutrient supply to adipocytes but also maintains a steady and mild shear stress on the cell membrane. It was found the perfusion-based culture conditions promoted faster growth of primary preadipocytes and stimulated greater adipogenesis compared to static culture condition. Adipocytes

cultured under perfusion systems produced more MCP-1 and IL-6, but less adiponectin. When stimulated with TNF- $\alpha$ , adipocytes expressed higher level of MCP-1 and IL-6, but lower level of adiponectin. No significant glucose uptake regulation was observed after treating the adipocytes with insulin in both static and perfusion-based culture. Our results demonstrate that perfusion-base culture has played a role in the adipocyte function particularly the secretion of adipokines. More future studies are required to unveil the mechanisms behind perfusion’s impact on adipocytes.

**Keywords** Adipocyte · Differentiation · Perfusion · Adipokines · Insulin resistance · TNF- $\alpha$  · Stimulation

## 1 Introduction

Due to the dramatic increase of obesity and its associated disorders including type 2 diabetes, hypertension, atherosclerosis, and cardiovascular diseases, adipose tissue has gained significant attention in recent years. Adipose has long been recognized as the main site of excess energy storage (Rosen and Spiegelman 2006). However, it becomes well accepted that adipose also function as an endocrine organ (Kershaw et al. 2004). Adipocytes, the dominant cellular component of adipose tissue, secrete adipokines, such as leptin, adiponectin, and monocyte chemoattractant protein-1 (MCP-1) (Balistreri et al. 2010; Kammoun et al. 2014). Adipokines regulate glucose homeostasis, affect the metabolic functions of other tissues (Clark et al. 2010; Davis and Scherer 2008; Oh et al. 2007; Sun et al. 2011), and modulate inflammatory responses (Meijer et al. 2011). Alteration of adipocyte function with associated dysfunctional production of adipokines is linked to the development of several important pathological conditions, including type 2 diabetes mellitus, cardiovascular

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disease, and cancers (Armani et al. 2010; Prieto-Hontoria et al. 2011; Percik and Stumvoll 2009). To better understand the molecular regulations of adipocyte function, specifically the metabolic and inflammatory response of adipocytes, physiologically relevant models that recapitulate the adipose physiology *in vitro* are required.

*In vivo*, cells are exposed to dynamic microenvironments, such as mechanical shear force, the composition and distribution of extracellular matrix, as well as the controlled release of soluble factors, which are crucial for controlling cell behavior. Perfusion-based cell culture has the potential to narrow the existing gap between *in vitro* and *in vivo* conditions by providing continuous supply of fresh nutrients and removal of waste, as well as applying *in vivo*-like mechanical shear stress/strain to cells. However, culturing of adipocytes in a perfusion environment is challenging. Adipocytes are buoyant due to lipid accumulation, which can be easily washed away by fluid flow. Recently, Abbott et al. described a perfusion-based cell culture device for long term adipogenesis by employing silk protein scaffolds as 3D framework in order to reduce loss of the fragile adipocytes under perfusion flow (Abbott et al. 2015). Godwin et al. developed a microfluidic device and used collagen gel to encapsulate primary adipocytes into a 3D environment to reduce cell lost during perfusion (Godwin et al. 2015). While these studies show promise towards recapitulating the adipose function *in vitro*, inoculation of adipocytes and monitor of cell behaviors in 3D still impose challenge.

In this work, we describe a perfusion-based cell culture device for human adipocyte culture which maintains continuous supply of fresh cell culture media and waste removal, while preventing cell washout. The device was constructed out of two vertically stacked compartments which were separated by a porous membrane. Adipocytes were cultured in the bottom compartment while the cell culture media were supplied through the upper compartment and subsequently infused through the porous membrane to the lower chamber. In this setting, the cells were not directly exposed to the main stream flow, therefore, subjected to a mild shear stress in a condition that is closer to *in vivo* environment (Rutkowski and Swartz 2007).

## 2 Experimental

### 2.1 Materials and reagents

Polydimethylsiloxane (PDMS) precursors, including Sylgard® 184 elastomer base and curing agent, were purchased from Dow Corning. Human recombinant insulin (Catalog # 12585014) and human recombinant tumor necrosis factor- $\alpha$  (TNF- $\alpha$ , Catalog # 10602HNAE25) were purchased from Life technologies. Human interleukin-6 (IL-6)

recombinant peptides (Catalog # 570808), LEAF™ purified anti-human IL-6 antibody (Catalog # 501110), biotin anti-human IL-6 antibody (Catalog # 501202), human adiponectin protein (Catalog # 558409), purified anti-human adiponectin antibody (Catalog # 528701), biotin anti-human adiponectin antibody (Catalog # 528803), HRP streptavidin (Catalog # 405210), and TMB solution (Catalog # 421501) were all purchased from Biolegend (USA). Phosphate buffer saline (PBS) was bought from Biowest (USA). Human MCP-1 Antibody Pair was purchased from Invitrogen (Catalog # CHC1013), which includes anti-human MCP-1, anti-human MCP-1 biotin, recombinant human MCP-1 and Streptavidin-HRP. Other chemicals were purchased from Sigma-Aldrich unless mentioned otherwise.

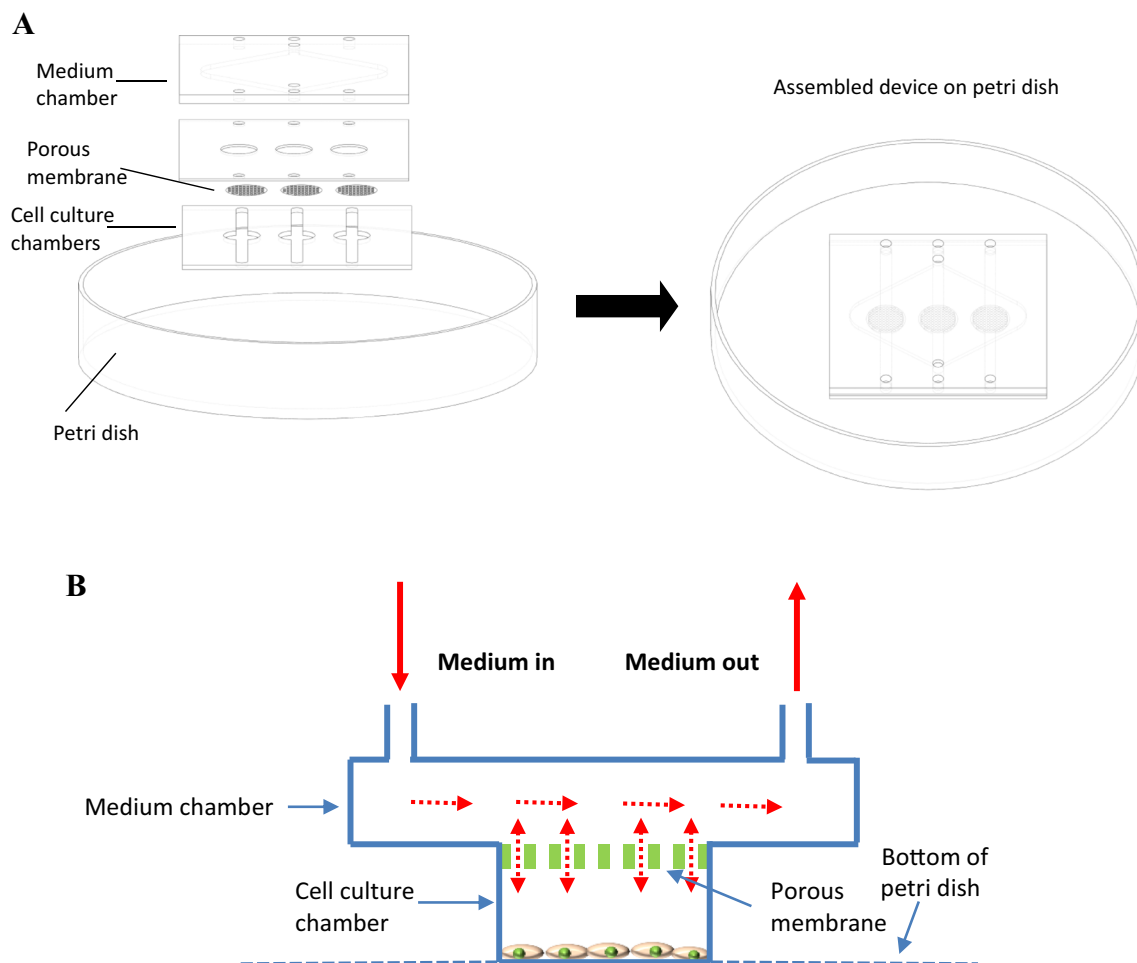
### 2.2 Device fabrication

Acrylic molds were fabricated using a CO<sub>2</sub> laser cutter (Universal laser). PDMS precursor solution was prepared by mixing elastomer and curing agent in a 10:1 ratio. After degasing, PDMS solution was poured onto the molds and cured overnight at 60 °C. Total three patterned sheets of PDMS were molded together to form the fluidic device (Fig. 1a), these are: (i) the bottom layer which contains three independent cell culture chambers with 8 mm diameter and 1 mm depth, (ii) the middle thin layer (0.5 mm thick) with three circular holes, which are aligned to the cell culture chambers to facilitate bonding of the porous membrane and separate the cell culture layer from top perfusion layer, (iii) the top layer containing a rhombus-shaped chamber with 1 mm depth which overlaps with the cell culture chambers for medium perfusion. Each cell culture chamber has a volume of 50  $\mu$ L, and the medium chamber is about 0.86 mL in volume. All fluidic ports were positioned in the upper layer to facilitate fluidic injection.

The bottom PDMS sheet was treated with O<sub>2</sub> plasma followed by application of a thin layer of (3-aminopropyl) triethoxysilane (APTES). After 5 min, the PDMS sheet was washed with deionized (DI) water and dried with nitrogen gas. The salinized PDMS was bonded to a petri dish at 60 °C overnight. Then a polyester membrane with 0.4  $\mu$ m pore size (Corning, Singapore) was bonded to the bottom PDMS layer using a thin layer of PDMS precursor. Then, the middle and top layers were bonded in a similar way. To enhance cell attachment, the petri dish was treated by a UV Ozone cleaner (ProCleaner™ Plus) before device assembly. The assembled device was immersed in 70% ethanol for 4 h and then immersed in sterilized PBS before cell seeding.

### 2.3 Cell culture

Cryopreserved human preadipocytes (Catalog # 802s-05a) were purchased from Cell Applications, Inc. (USA). After



**Fig. 1** **a** Assembly of perfusion device for adipocyte culture. **b** Schematic illustration of medium perfusion through the fabricated device (a cross-sectioned view), only one cell culture chamber was shown here for the reason of simplification. Figures were not drawn to scale

thawing, preadipocytes were sub-cultured in preadipocyte growth medium (Catalog # 811–500, Cell Applications) and passaged. Preadipocytes, from passage 4, were seeded into the devices and maintained in preadipocyte growth medium at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. To allow attachment, cells were kept in a static culture overnight. After 24 h, the device was connected to the perfusion apparatus which comprises of a syringe pump (Harvard Apparatus, USA) and a culture media reservoir. The culture media reservoir was kept inside the cell culture incubator and connected to the device through silicone tubing while the outlet was connected to a syringe. A negative pressure was applied by the syringe pump, so that cell medium was continuously withdrawn from the reservoir and injected into the device. Two flow rates have been applied, 5 nL/s and 15 nL/s. Static cell culture was also conducted in parallel as a control, where culture medium was refreshed every two days. To induce cell differentiation, once preadipocyte formed a confluent layer, the preadipocyte growth medium was replaced with adipocyte differentiation medium (Catalog # 811D-250, Cell Applications). Fresh adipocyte maintenance medium

(Catalog # 811M-250, Cell Applications) was continuously perfused through the upper chamber for another 3–4 days before treatment.

## 2.4 Cell proliferation

Cell proliferation was measured using Cell Counting Kit 8 (CCK-8, Sigma). After a certain time of culture, the medium was removed, and 100 µL of fresh medium with 10% of CCK-8 was added to each cell culture chamber. After 1 h, the medium was collected, and 10 µL of 1% sodium dodecyl sulfate (SDS) was added to stop color development. Absorbance at 450 nm was measured using Enspire® Multimode Plate Reader (PerkinElmer). A standard curve (CCK-8 absorbance versus cell numbers) was obtained by seeding cells with known count (counted using Luna™ cell counter) and measuring the absorbance of CCK-8 after attachment. Cell numbers during culturing were calculated based on the standard curve.

## 2.5 Lipid droplet staining

After differentiation, adipocytes were fixed in 2% paraformaldehyde (PFA). Lipid droplets were stained with Oil Red O solution containing 2 parts of DI H<sub>2</sub>O and 3 parts of Oil Red O stock (0.5% in isopropanol). After washing with DI H<sub>2</sub>O, bright-field images were acquired with an upright microscope (Olympus, BX3-CBH, Japan). To quantify the stain, 100% isopropanol was used to extract the stain and absorbance was measured at a wavelength of 492 nm.

## 2.6 Cell treatment and immune assays

After cell differentiation (cells differentiated for 14 days), the cell culture medium was switched to the adipocyte maintenance medium and supplied for 3–4 days. Then the medium from the top chambers were discarded and the perfusion was stopped. 100  $\mu$ L of fresh adipocyte maintenance medium was added to each cell culture chambers and incubated for 24 h. Then the medium was collected and stored at -20 °C for ELISA testing. TNF- $\alpha$  stimulation was carried out on the same set of cells by applying adipocyte maintenance medium with 10 ng/mL of TNF- $\alpha$  for 24 h. Then the medium was collected at the end of treatment and stored at -20 °C for ELISA testing. MCP-1, IL-6, and adiponectin in the medium were measured using ELISA assay according to the manufacturer's instructions.

## 2.7 Glucose uptake assay

Glucose uptake by the adipocytes was detected using a glucose uptake cell-based assay kit (Cayman Chemicals, Ann Arbor, MI, USA). The kit employs 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG), a fluorescent-labeled deoxyglucose analog, as a probe for the detection of glucose uptake by adipocytes. Differentiated adipocytes were maintained in adipocyte maintenance medium for 3–4 days. Then cells were treated with adipocyte maintenance medium without insulin (Catalog # 811-Mi-250, Cell Applications) overnight, followed by adipocyte starvation medium (Catalog # 811S-250, Cell Applications) for 4 h. Cells were washed 3 times with PBS. After that, cells were incubated in Krebs-Ringer-Phosphate-HEPES (KRPH) buffer (20 mM HEPES, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 136 mM NaCl, and 4.7 mM KCl, pH 7.4) with 2% BSA for 40 min. Insulin stimulation was carried out via incubation in insulin solution (8  $\mu$ g/mL in KRPH buffer with 2% BSA) for 30 min. 2-NBDG was added at a concentration of 200  $\mu$ g/mL, and incubated for 1 h. Cells were washed with assay buffer. Fluorescent images were taken by an upright Olympus microscope (Olympus BX3) using a fluorescein filter with excitation/emission of 485/535 nm. Fluorescent

intensity from the obtained images was analyzed using Image J software.

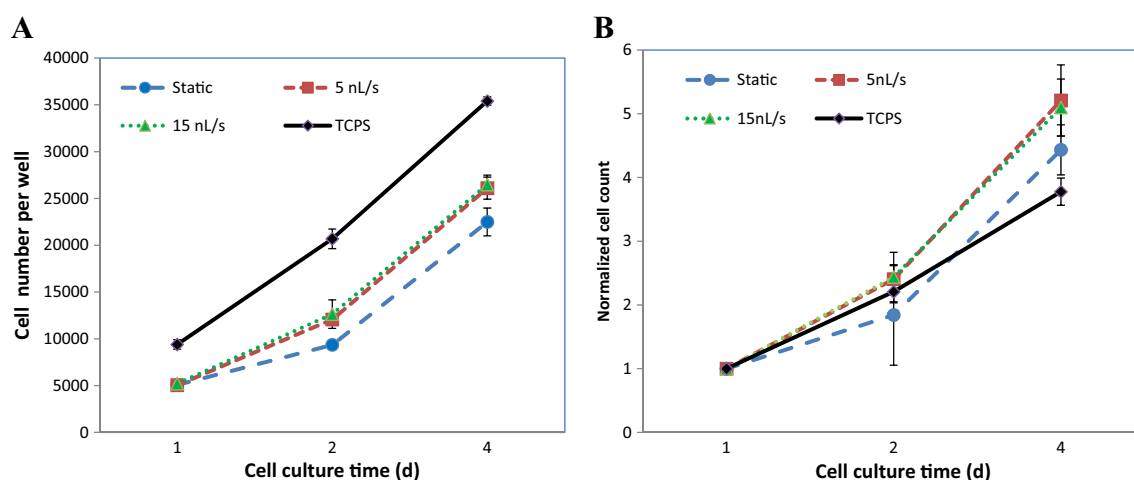
## 2.8 Statistics

For CCK-8 assay, Oil Red O, MCP-1, IL-6 and adiponectin measurements, data were collected from at least triplicate cell culture and presented as means  $\pm$  standard deviation. For glucose uptake assay, fluorescent images were taken from three parallel cultures for each condition, and fluorescent intensity from at least 100 cells was averaged. A student's *t* test was performed between two different groups. *P* < 0.05 was considered significant.

## 3 Results and discussions

In this paper, we have characterized the differentiation and stimulation of human adipocytes in a dynamic cell culture system. The large size of the differentiated adipocytes and the secreted hydrophobic metabolites may lead to channel blockage and consequently cell culture failure (Clark et al. 2009). Therefore, to avoid channel blockage, we fabricated a fluidic device with relatively large channels (about 2.5 mm) and chambers, as shown in Fig. 1a. The diameter and depth of each cell culture chamber are 8 mm and 1 mm, respectively. Each device comprises an array of 3 cell culture chambers which allows three parallel experiments from the same culture/stimulation conditions. A mild shear stress on the adipocyte membrane was achieved by adopting a through-membrane perfusion setup during the course of cell culture (Fig. 1b), thus minimizing the chance of cell detachment from the surface. In addition, the use of a dedicated perfusion chamber that is separated from the cell culture chamber by a porous membrane inhibited the gas bubble formation which could severely disturb the cell culture environment.

Preadipocytes are adipogenic precursor cells which can proliferate and differentiate into adipocytes (Hemmrich et al. 2005). Due to the limited supply of primary mature adipocytes, preadipocytes are usually used. To investigate the effect of perfusion on cell proliferation and differentiation, culture media were supplied in continuous flow at two different flow rates, which are 5 nL/s and 15 nL/s. In parallel, static culture was also carried out through which a 0.86 mL of fresh culture medium was added to the top chamber every two days for each device. When a flow rate of 5 nL/s was used in perfusion-based culture, a total media volume of 0.86 mL would be replaced every two days, which is the same as the volume used in static culture. It should be noted that, in perfusion culture, fresh medium is constantly replenished, which provides a continuous supply of nutrients and ensures the removal of cell culture waste including possible soluble toxins. As shown in Fig. 2a, cell numbers steadily increased



**Fig. 2** Growth charts for preadipocytes in devices under static or perfusion conditions. **a** Cell numbers versus culture time. Cell number was measured using CCK-8. **b** Cell numbers were normalized to day 1 count. TCPS represents 96-well tissue culture plates

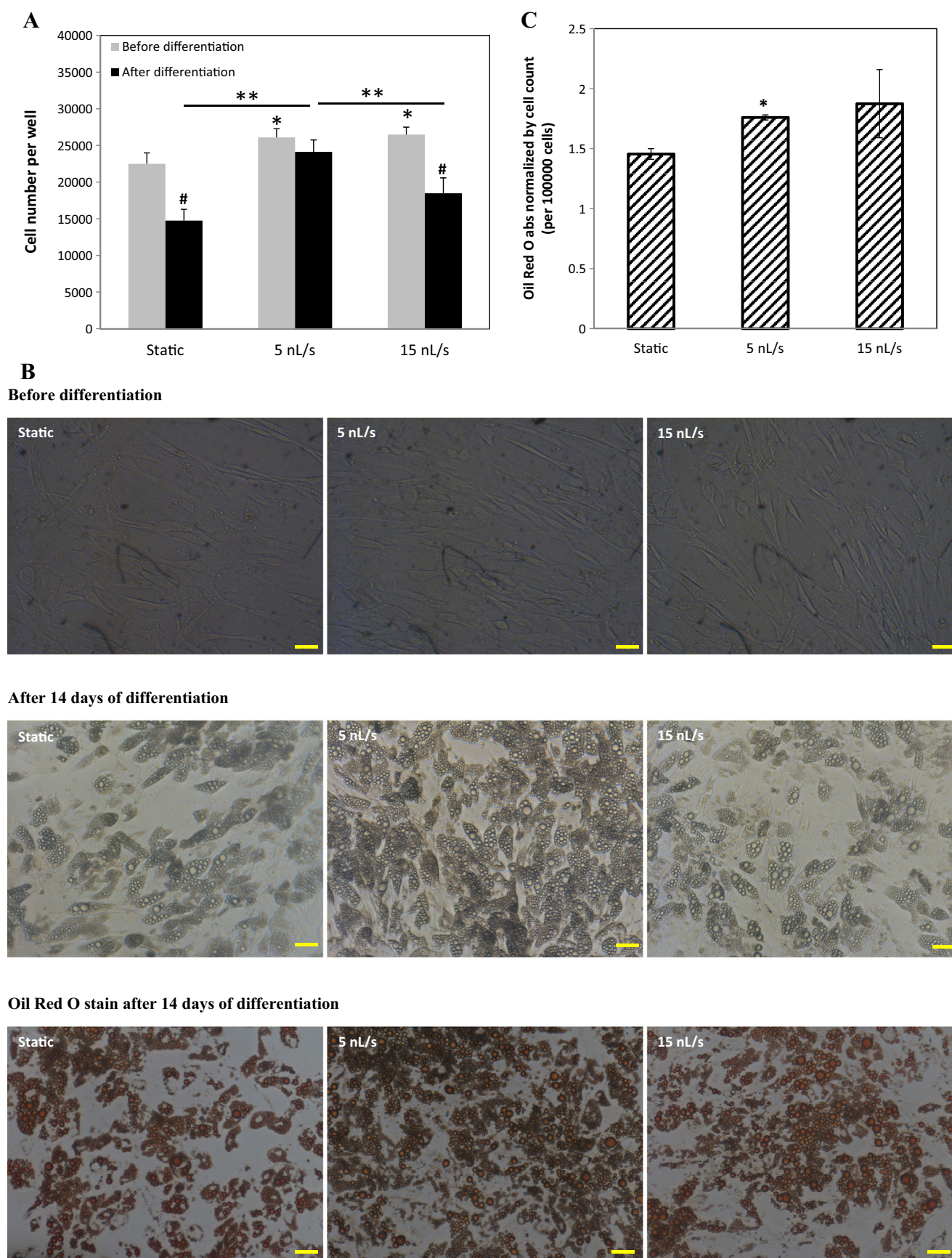
during the first four days of culture in both static and perfusion-based culture, following the same trend as cells cultured on 96-well tissue culture plates (TCPS). However, it was observed that the proliferation rate (Fig. 2b) of the preadipocytes under perfusion culture is a little bit faster than that under static cultures either on the device or on TCPS. There is no difference in cell proliferation between 5 nL/s and 15 nL/s perfusion rates.

Once preadipocytes formed a fully confluent layer, the preadipocyte growth medium was replaced with a differentiation medium. Under differentiation condition, preadipocyte gradually enlarged and started to display vesicles (Fig. S1). Differentiated adipocytes form lipid droplets that can be considered as a biomarker of cell differentiation and can be clearly visualized by Oil Red O staining (Tenstad et al. 2010). After 14 days of differentiation, cells were counted using CCK-8. Cell number decreased after differentiation in all static and perfusion cultures, with 5 nL/s perfusion culture has the least cell loss (Fig. 3a). After differentiation, the number of cells was higher in the perfusion culture than that in static culture, with the highest number in the perfusion culture with a flow rate of 5 nL/s (Fig. 3a). As shown in Fig. 3b, cells cultured under a 5 nL/s perfusion rate had a significantly higher amount of vesicles than that under static culture or a 15 nL/s perfusion rate. Oil Red O stains confirmed that the vesicles were fat droplets. Some cells showed no lipid droplet, indicating that not all preadipocytes were differentiated into adipocytes. The absorbance of Oil Red O staining was then normalized to cell number. Under perfusion culture, there were higher percentages of differentiated adipocytes, as indicated by the higher normalized Oil Red O absorbance (Fig. 3c). It is clear that the perfusion conditions, with constant nutrient supply and waste removal, are favorable for adipogenic differentiation of preadipocytes.

Adipocytes secrete various biologically active factors, such as MCP-1, IL-6, TNF- $\alpha$ , and adiponectin, which actively modulate the inflammatory response in adipose tissue (Fried

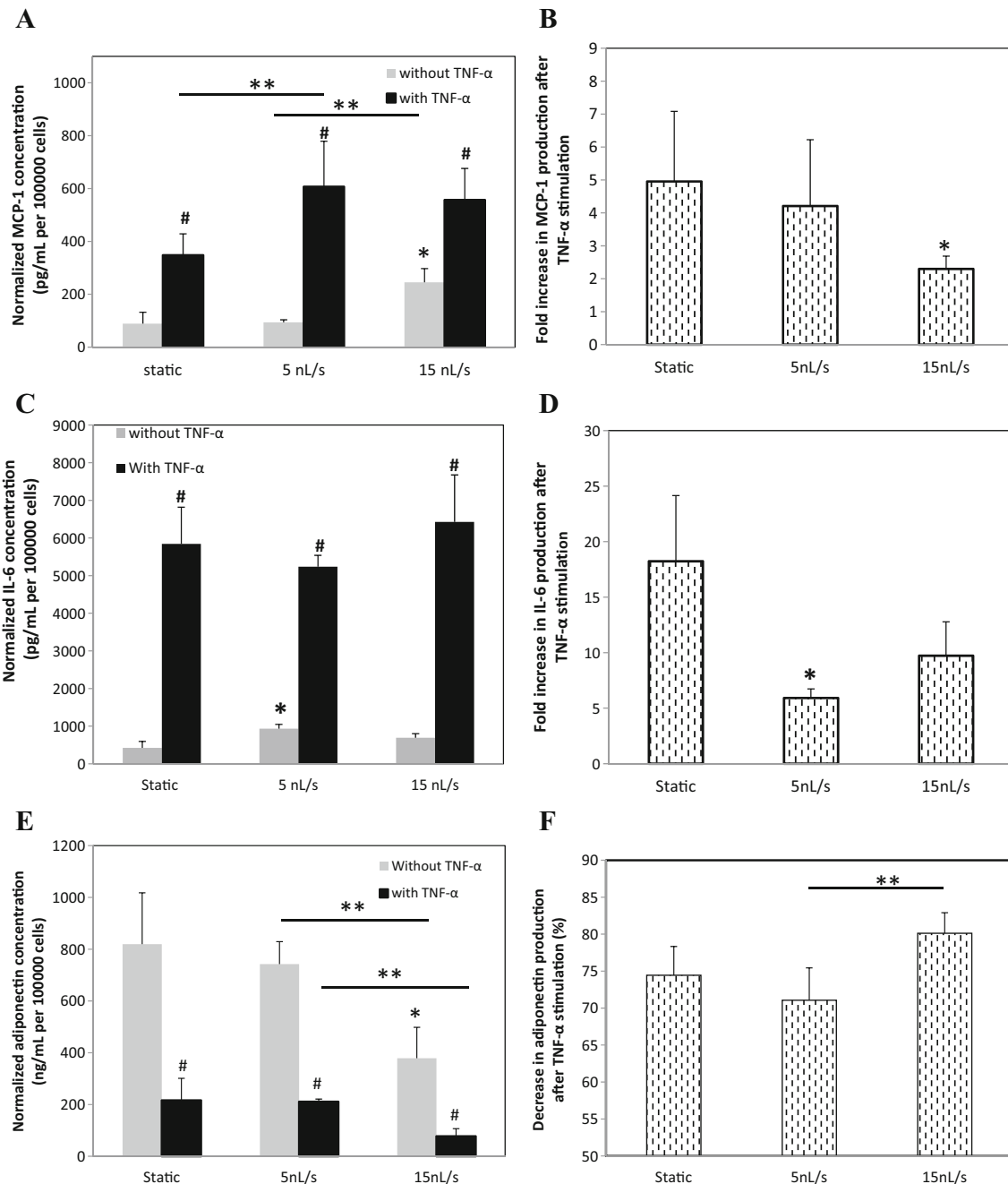
et al. 1998; Mohamed-Ali et al. 1997; Sartipy and Loskutoff 2003; Xu et al. 2003). MCP-1 plays an important role in regulating the migration and infiltration of monocytes/macrophages (Kanda et al. 2006). IL-6, a pro-inflammatory cytokine, has been shown to impair insulin signaling in 3T3-L1 adipocytes (Rotter et al. 2003). TNF- $\alpha$  was also reported to impair insulin receptor signaling (Stephens et al. 1997; Qi and Pekala 2000), stimulate the release of circulating nonesterified fatty acids from adipocytes (Guilherme et al. 2008) and in consequence contribute to insulin resistance. TNF- $\alpha$  was detected at an elevated level in adipose tissue of obese humans (Hotamisligil et al. 1993; Hotamisligil and Spiegelman 1994; Hotamisligil et al. 1995). It was hypothesized that, macrophages infiltrated into the adipose tissue is responsible of the increase level of TNF- $\alpha$  secretion (Xu et al. 2003; Nitta and Orlando 2013). Adiponectin is an anti-inflammatory hormone. It is considered as a differentiation marker in adipocytes and plays a role in glucose homeostasis (Wang et al. 2005; Kadowaki et al. 2006; Shetty et al. 2009). Secretion of adiponectin was reported to be suppressed in insulin-resistant and obese individuals (Weyer et al. 2001).

After the successful differentiation, the immune status of the differentiated adipocyte was characterized by measuring several adipokines, including MCP-1, IL-6, TNF- $\alpha$ , and adiponectin, in the cell culture supernatant. The amount of TNF- $\alpha$  in medium from cultured adipocytes under both perfusion and static conditions was negligible (data not shown), whereas MCP-1, IL-6, and adiponectin were all detected (Fig. 4). Higher expressions of MCP-1 and IL-6 were detected in the perfusion-based culture (both 5 nL/s and 15 nL/s) than in static culture (Fig. 4a and c). In contrast, the expression of adiponectin was lower in the perfusion-based culture comparing to that in static culture (Fig. 4e). MCP-1 and IL-6 expression significantly increased when adipocytes were stimulated with TNF- $\alpha$  (Fig. 4a and c). On the other hand, adiponectin expression was down-regulated under TNF- $\alpha$  stimulation (Fig. 4e).



**Fig. 3** **a** Quantification of cell number for adipocytes in static and perfusion devices just before differentiation and after 14 days of differentiation. (\*)  $P < 0.05$ , before differentiation, compared with static culture, (\*\*)  $P < 0.05$ , between two groups indicated by the line, (#)  $P < 0.05$ , compared between the same culture condition before and after differentiation. **b** Bright-field images of cells before differentiation, after 14 days of differentiation, and stained with Oil Red O after 14 days of differentiation under static and perfusion conditions. Scale bar = 50  $\mu\text{m}$ . **c** Normalized Oil Red O staining. (\*)  $P < 0.05$  compared with static culture

It should be noted that, the up-regulated expression of MCP-1 and IL-6, and down-regulated expression of adiponectin, under static culture when stimulated with  $\text{TNF-}\alpha$ , are consistent with previously reported studies (Nitta and Orlando 2013; Gonzales and Orlando 2008; Rotter et al. 2003; Hector et al. 2007). The  $\text{TNF-}\alpha$  associated up-regulation of MCP-1 and IL-6 can be attributed to the



**Fig. 4** **a** MCP-1, **c** IL-6, and **e** adiponectin release from adipocytes under static and perfusion conditions without or with the stimulation of  $\text{TNF-}\alpha$ . Folds of increase in MCP-1 (**b**) and IL-6 (**d**) production after stimulation was calculated via dividing the expression amount after  $\text{TNF-}\alpha$  by that before  $\text{TNF-}\alpha$ . **f** Decrease in adiponectin production was calculated via

dividing the decreased amount in expression by the expression amount before  $\text{TNF-}\alpha$ . (\*)  $P < 0.05$ , without  $\text{TNF-}\alpha$  stimulation, compared with static culture; (#)  $P < 0.05$ , compared between the same culture condition without and with  $\text{TNF-}\alpha$ ; (\*\*)  $P < 0.05$ , between two groups indicated by the line

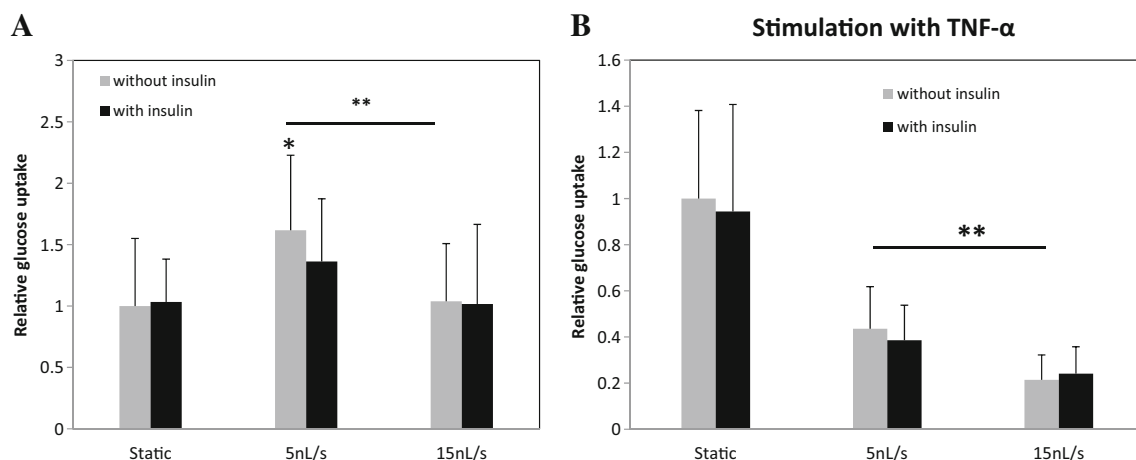
induced inflammatory reaction in adipocytes by TNF- $\alpha$ . In general, TNF- $\alpha$  stimulation results in the same trend of change in adipokines (MCP-1, IL-6, and adiponectin) expression in both static and perfusion-based cultures, i.e. increased the secretion of MCP-1 and IL-6 and decreased secretion of adiponectin. Compared with static culture, the folds of increase in MCP-1 and IL-6 secretion by adipocytes under perfusion culture were lower (Fig. 4b and d), whereas the decrease in adiponectin production was similar (Fig. 4f).

The glucose uptake of adipocytes was evaluated by employing the commercially available 2-NBDG, a fluorescently-labeled deoxyglucose analog. The fluorescent images of adipocytes taking 2-NBDG in both static and perfusion conditions are shown in Fig. S2. First, we evaluated the effect of perfusion on glucose uptake by the adipocytes. As shown in Fig. 5a, the basal glucose uptake by the adipocytes is the highest in cells under perfusion with a flow rate of 5 nL/s. However, when stimulated with TNF- $\alpha$ , cells in the perfusion cultures showed lower levels of basal glucose uptake than those in the static culture (Fig. 5b). Furthermore, the glucose uptake was even down-regulated when increasing the flow rate from 5 nL/s to 15 nL/s. Second, we evaluated the effect of insulin on glucose uptake. The regulation of glucose transport in fat by insulin is well-known (Leto and Saltiel 2012). However, no significant change in glucose uptake was observed in our experiments under both static and perfusion based culture when stimulated with insulin. For most groups, glucose uptake levels did not change much after insulin stimulation (Fig. 5a and b). These results were also confirmed in parallel experiments using the conventional 96-well tissue culture plate (data not shown). We speculate that the *in vitro* differentiated adipocytes have developed insulin resistance or the adipocytes used in our experiments were not sensitive to the 2-NBDG glucose analog which will be further investigated. However, the lower basal glucose uptake under perfusion

culture after TNF- $\alpha$  stimulation may be related to the higher levels of MCP-1 expression. A short term (6 h) MCP-1 treatment has been reported to induce a significant reduction in basal glucose uptake in adipocytes (Sartipy et al., 2003).

We have demonstrated that cell culture under perfusion conditions accelerate and enhance adipocyte differentiation, which can be attributed to the continuous and controlled nutrition supply by fresh medium perfusion. Surprisingly, adipocytes in perfusion-based culture were found to express more pro-inflammatory cytokines, MCP-1 and IL-6, and less anti-inflammatory adiponectin compared to those in static culture. Different perfusion rates had induced differential effects on adipokine secretion, e.g., higher perfusion rate (15 nL/s) resulted in significantly higher amount of MCP-1 and lower amount of adiponectin production than lower perfusion rate (5 nL/s), indicating a higher degree of inflammatory reaction under higher perfusion rate. Perfusion provides a steady mass transfer from the flow stream (top) into the cell culture chamber (bottom) which may emulate the local mass transfer gradient *in vivo*, thus more nutrients are provided to the cells under perfusion. With the increase of perfusion rate, even more nutrients are supplied. The induced inflammatory reaction by perfusion culture may due to more nutrient supply. It was reported that nutrient overload may trigger adipose tissue immune response (Wellen et al. 2007). It is also noted that cell loss during preadipocytes differentiation period was significantly higher under perfusion culture of 15 nL/s than 5 nL/s (Fig. 3a). This may be caused by the increased MCP-1 secretion of adipocytes cultured under a higher perfusion rate (Fig. 4a). Negative correlation between cell viability and MCP-1 secretion has been reported (Garcia-Diaz et al. 2011).

In addition, the perfusion flow imposes mild mechanical shear stresses on the adipocytes membrane which also could contribute in modulating the adipocyte responses. Fig. S3 shows the shear calculated stress using the Finite Element



**Fig. 5** Relative 2-NBDG uptake as measured by fluorescent intensity on cultures without TNF- $\alpha$  (a), and after TNF- $\alpha$  stimulation (b). Data shown here are normalized to static culture without insulin stimulation. (\*)

$P < 0.05$ , perfusion cultures without insulin stimulation, compared with static culture; (\*\*)  $P < 0.05$ , between two groups indicated by the line

Methods (COMSOL MultiPhysics). A maximum shear stress of  $\sim 7.5 \times 10^{-4}$  dynes/cm<sup>2</sup> was calculated when a flow rate of 15 nL/s was used. When the flow rate decreased to 5 nL/s, maximum shear stress decreased to  $2.5 \times 10^{-4}$  dynes/cm<sup>2</sup>. It is worth noting that adipocytes *in vivo* are subject to interstitial shear stress. Interstitial shear stress, induced by extravascular fluid flowing through a three-dimensional extracellular matrix, is extremely small ( $\leq 0.01$  Pa) (Kim et al. 2014). However, it can affect cell differentiation, migration, and other functions (Rutkowski and Swartz 2007). Adipocytes have been cultured under perfusion conditions with rather high flow rates ranging from 0.3  $\mu$ L/min to 80  $\mu$ L/min (Ni et al. 2008; Clark et al. 2009; Abbott et al. 2015; Godwin et al. 2015). In one study, fluid shear stress of 1 Pa (much higher than interstitial level) was applied during adipocyte maturation period, and lipid droplet accumulation was reported decreasing due to fluid shear stress (Choi et al. 2016). However, the influence of low interstitial levels of fluid shear force on adipocytes has not been investigated. Compare static culture with 5 nL/s perfusion culture, significantly higher differentiation efficiency was observed for perfusion culture. Low fluid shear stress may have a different effect on adipocytes compared with a high fluid shear stress. However, since the way of nutrient supply is also different in both cultures, the exact effect of low fluid shear stress on adipocytes requires further clarification.

In general, interstitial cells such as adipocytes, fibroblasts, and immune cells need physiological fluid flow for nourishment and mechanotransduction *in vivo* (Swartz and Fleury 2007). Perfusion culture system provides a close mimic to the *in vivo* interstitial flow conditions. With perfusion, not only the transport of solute especially large molecules such as proteins is facilitated by convection, but also fluid shear stress is applied to cell surface. However, the perfusion rate should be carefully tuned, thus a more physiologically relevant cell culture model can be obtained.

## 4 Conclusions

Human adipocytes have been cultured, differentiated and stimulated in a perfusion device. The double-layered fluidic structure and the porous membrane allow continuous perfusion of culture media and at the same time facilitate the application of a mild shear stress on the membrane of adipocytes. Preadipocytes were shown to proliferate faster under perfusion than static conditions. Continuous perfusion of nutrient stimulated greater adipogenesis. More MCP-1 and IL-6 and less adiponectin expression were observed under perfusion culture compared to static culture. Adipocytes showed response to TNF- $\alpha$  stimulation under both static and perfusion conditions. However, glucose uptake regulation by insulin was negligible in the adipocytes under study. To our

knowledge, this is the first report on the effect of perfusion-flow on the adipocyte adipokine expression. More systematic studies are required to explore the mechanism of insulin function in the adipocytes as well as the influence of immune cells in insulin sensitivity, which may help improve our understanding the role of inflammation in insulin resistance.

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