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Electric impedance sensing in cell-substrates for rapid and selective multipotential differentiation capacity monitoring of human mesenchymal stem cells

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ABSTRACT

Biosensor systems which enable impedance measurements on adherent cell layers under label-free conditions are considered powerful tools for monitoring specific biological characteristics. A radio frequency identification-based sensor platform was adopted to characterize cultivation and differentiation of human bone marrow-derived multipotent stem cells (bmMSC) over periods of up to several days and weeks. Electric cell-substrate impedance sensing was achieved through fabrication of sensitive elements onto glass substrates which comprised two comb-shaped interdigitated gold electrodes covering an area of 1.8 mm × 2 mm. The sensing systems were placed into the wells of a 6-well tissue culture plate, stacked onto a reader unit and could thus be handled and operated under sterile conditions. Continuous measurements were carried out with a sinusoidal voltage of 35 mV at a frequency of 10 kHz.

After seeding of human bmMSC, this sensor was able to trace significant impedance changes contingent upon cell spreading and adhesion. The re-usable system was further proven suitable for live examination of cell-substrate attachment or continuous cell monitoring up to several weeks. Induction of either osteogenic or adipogenic differentiation could be validated in bmMSC cultures within a few days, in contrast to state-of-the-art protocols, which require several weeks of cultivation time. In the context of medical cell production in a GMP-compliant process, the here presented interdigitated electric microsensor technology allows the documentation of MSC quality in a fast, efficient and reliable fashion.

1. Introduction

Mesenchymal stem cells (MSC), which are residing throughout the human body, are currently employed in various biotechnological and biomedical applications and are considered of high importance for future cutting-edge clinical practice (Kassem and Abdallah, 2008). However prior to most cell-based therapeutic and tissue-engineering applications, primary cell isolates have to be culture-expanded. Quality control measures are immune phenotyping by flow cytometry and the concomitant assessment of the cells' multipotential differentiation capacity (Dominici et al., 2006). In this context, two most prominent lineages are the formation of specific precursors for bony and fatty tissues. In the latter context, innovative devices, which allow the quantification of cell-fate changes in cultured multipotential stem cells, preferably by label-free means, is much in demand, since particularly in the case of MSC, their potential to self-renew and their respective regenerative vigor are well acknowledged as highly valuable assets in tissue repair (Tolar et al., 2011). Moreover, MSC are contemplated for future cutting-edge clinical applications in the treatment of neurodegenerative and autoimmune disease (D'Agostino et al., 2010; Salem and Thiemermann, 2010; Uccelli and Mancardi, 2010; Uccelli and Prockop, 2011), suffice it to say that potential outcomes of MSC-based therapies are greatly dependent on the quality of the applied cells.

To date there is no instrument which allows the assessment of MSC stemness in a standardized fashion. During *in vitro* cultivation the quality of MSC ceases steadily (Fehrer et al., 2006; Stolzing et al., 2008; Wagner et al., 2010), this fact certainly being a major limitation for many novel technological and clinical approaches (Caplan, 2007; Roobrouck et al., 2008). Biosensors that enable impedance measurements are considered applicable for examination of proliferation of cultivated cells (Angstmann et al., 2011;

Abbreviations: 7AAD, 7-amino-actinomycin D; MEM, modified essential medium; MSC, mesenchymal stem cells; PDMS, polydimethylsiloxane; RFID, radio frequency identification.

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Bagnaninchi and Drummond, 2011; Hildebrandt et al., 2011; Park et al., 2011). In this contribution we report the implementation of a radio frequency identification (RFID)-based sensor platform, also called '*CellMonitor*', for the *in vitro* assessment of osteogenic and adipogenic differentiation of human bone marrow-derived multipotent stromal cells. This system enables continuous monitoring of cells under standard culture conditions. More than that, also the differentiation potential of MSC towards osteogenic or adipogenic fate can be determined rapidly and reliably.

2. Materials and methods

2.1. Reagents and chemical compounds

All reagents were purchased from Sigma–Aldrich Austria (Vienna, Austria) unless otherwise stated.

2.2. Impedance measurements

MSC adhesion and differentiation into the osteogenic or adipogenic lineage was monitored with the RFID-based sensor platform *CellMonitor*. The sensor system has been constructed and assembled for use in a sterile and humidified environment (Wissenwasser et al., 2010b). Comparable to cultivation in cell culture plastic, cells were seeded onto the individual, re-usable sensors, which were fabricated on 0.5 mm AF45 glass substrates (Schott AG, Germany) and mounted with a polydimethylsiloxane (PDMS) funnel to provide a well appropriate for cell cultivation. The independent and battery-free sensing units were placed into a 6-well tissue culture plate, stacked onto the reader unit and subsequently positioned inside a cell culture incubator. Continuous measurements were carried out with a sinusoidal probe voltage of 35 mV rms. Regarding cell proliferation, a signal frequency of 10 kHz has been reported to specifically yield optimal results (Ehret et al., 1997). Detailed technical specifications of the instrument have been described previously (Wissenwasser et al., 2011). Data acquisition and command transfer were achieved with the aid of proprietary software.

2.3. MSC isolation, cultivation and differentiation

Human MSC were isolated, cultivated, and characterized as described previously (Fehrer et al., 2007). Briefly, MSC were harvested from collagenase-treated bone biopsies of healthy individuals. Cells were cultivated at 37 °C, 3% O_2, and 5% CO_2 in minimum-essential-medium (MEM) supplemented with 20% fetal calf serum, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all Gibco, Invitrogen, Austria). To monitor differentiation, 50,000 MSC from passage 2 (total volume of 360 μl) were seeded at 20% O_2 and allowed to adhere to the sensor-equipped substrate. After 48 h, medium was replaced and loosely adhering cells were discarded. 24 h thereafter, control cells were kept in regular MEM growth medium, whilst in parallel, differentiation was

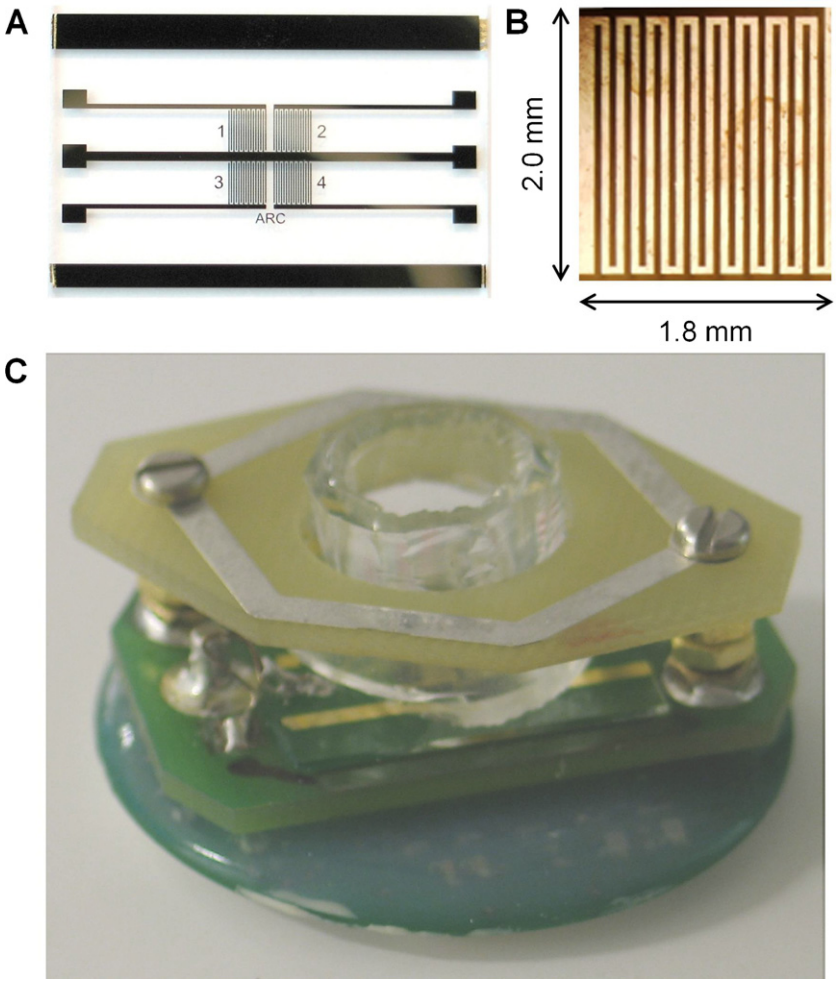

Fig. 1. Sensor design. (A) The sensor carries 4 sensitive elements comprising an overall size of 15 mm × 18 mm. (B) A single element of 1.8 mm × 2 mm is composed of interdigitating fingers with both a width and a gap of 50 μm. (C) The assembled sensor unit with its corresponding electronic tag fits into 6-well microtiter plates.

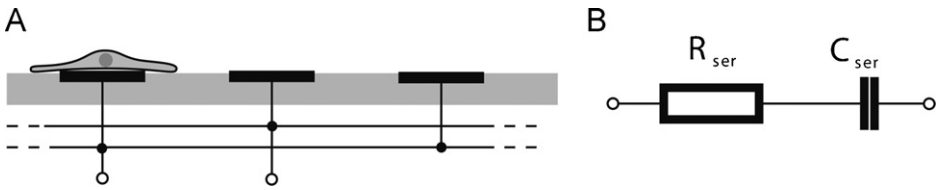

Fig. 2. Equivalent circuit model. (A) Schematic section through a sensor field showing one cell in direct contact with the sensor surface. The interdigitated electrodes (black bars) are fixed onto a glass substrate (grey). (B) Equivalent circuit model. Variations of the ohmic resistor (R_{ser}) are a measure for the number of adhered cells, whereas variations of the capacitor C_{ser} are less pronounced.

induced as follows: for osteogenesis MEM was supplemented with 1 mM β-glycerol phosphate, 100 nM dexamethasone, and 500 nM ascorbate-2-phosphate, while for the induction of adipogenesis, 60 μM indomethacin, 1 μM dexamethasone, 500 nM hydrocortisone, 50 μM 3-isobutyl-1-methylxanthine were added. Media were changed 3, 5, and 7 days after initiation of differentiation.

2.4. Validation of cell viability

An equal number of cells were seeded per sensor well either with or without current flow. To estimate the impact of the applied electric field during impedance measurement phases, MSC were cultivated under standard conditions as described above. After 6 days, cell viability was determined by means of 7-amino-actinomycin D staining (7AAD, 10 μg ml^{-1}) and concomitant counterstaining with Hoechst 33342 (25 μg ml^{-1}) in phosphate-buffered saline adjusted to pH 7.4 (PBS). The number of 7AAD-positive cells was accounted in 8 representative, non-overlapping regions comprising equivalent microscopic fields bearing comparable cell numbers.

2.5. Quantitative reverse-transcription PCR analysis

For quantitative PCR analysis, the MSC were seeded into conventional culture dishes at a density of approximately 1000 cells cm^{-2}. The cells were grown to a confluent layer in growth medium and then differentiated into both lineages as described above. Total RNA was isolated using the RNeasy Plus Mini Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). cDNA synthesis as well as PCR analysis was performed as described earlier. Transcript levels for alkaline phosphatase (ALPL) and secreted phospho protein 1 (SPP1, osteopontin) were determined in the case of osteogenic differentiation, while in the case of adipogenesis-induced cells lipoprotein lipase (LPL) and fatty acid binding protein 4 (FABP4) were utilized as specific markers. Messenger RNA expression levels were determined by calculating $\Delta\Delta C_q$ values relative to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bustin et al., 2009). Sequences for gene-specific primer pairs were taken from Laschober et al. (2011); in the case of GAPDH, the respective forward and reverse primer were ATG TTC GTC ATG GGT GTG and GTC TTC TGG GTG GCA GTG.

2.6. Assessment of alkaline phosphatase enzymatic activity

MSC were cultured in 24-well plates at a density of 15,000 cells cm^{-2} in regular growth medium, or the respective differentiation media. After appropriate treatment (see above) and before harvesting the cells with the aid of a cell scraper, the cultures were washed with 1 ml Dulbecco's modified PBS (Gibco, Invitrogen, Austria). The cells were lysed upon addition of 100 μl 50 mM Tris pH 7.4 containing 0.5% Nonident P40 Substitute (Fluka, Germany). After homogenization through vigorous pipetting, the debris was removed by centrifugation. Alkaline phosphatase enzyme activity was measured in 96-well plates by mixing 10 μl cell homogenate with 150 μl CDP Star detection reagent (GE Healthcare, UK). Chemiluminescence was monitored with the aid of a Wallac Victor^2 instrument (HVD-PerkinElmer, Austria).

3. Results and discussion

Over the last years, MSC have been celebrated a much sought-after remedy, not only in the wide context of anti-aging and regenerative medicine, but in particular also for combating autoimmune or graft-*versus*-host disease (Lepperdinger, 2011). Linked to

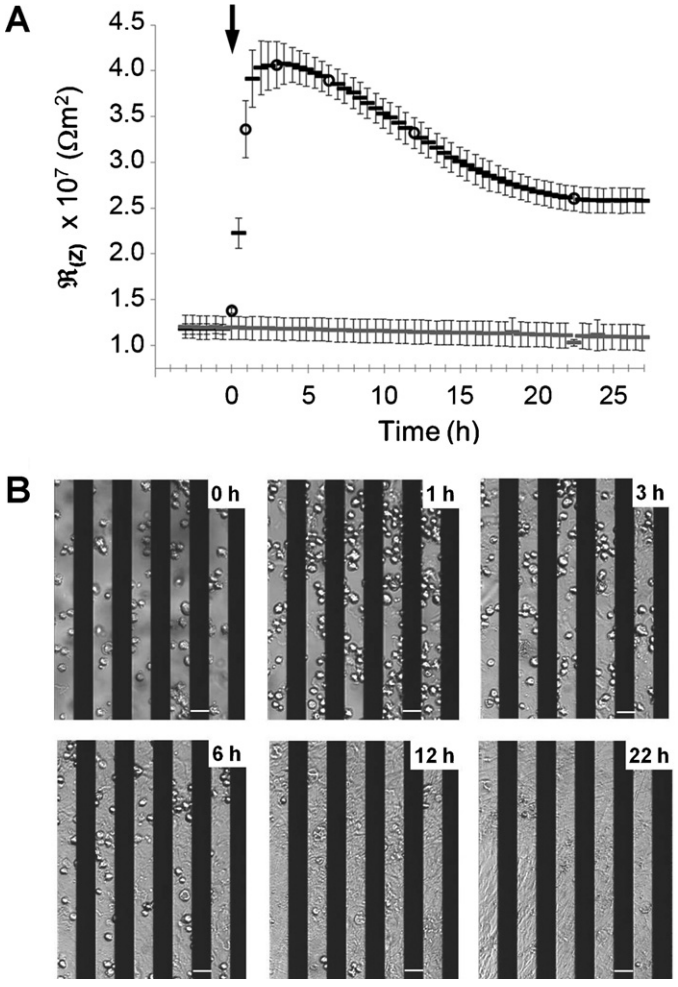

Fig. 3. Impedance monitoring of cell seeding, adhesion and spreading. (A) Changes in impedance after seeding of 50,000 MSC per well on sensor surfaces (black, $n = 6$) and controls (only cell culture medium, grey, $n = 4$) were monitored over a period of 27 h (sensing interval = 0.5 h). Arrow indicates the time point when cells were added. (B) Micrographs of the sensor surface after cell seeding were taken at indicated time points (open circles in A) with the aid of a live cell imaging system (JuLI, PAA Laboratories GmbH, Austria). Mean values ± SD are depicted. White bars on the IDES metallization indicate 50 μm.

that, it is generally accepted that standardized MSC quality controls need to be carefully specified in order to assure patients' safety as well as to incessantly advance existing and novel approaches in this cutting-edge research field (Wagner et al., 2010). In line with this, cleverly devised biosensor systems designed for cell biology have to be adopted for stem cell research; notably sensor technology suitable for impedance measurements on adherent cell layers are useful and suitable tools for sensitively quantifying specific biological characteristics (Flanagan et al., 2008; Keese et al., 2002).

3.1. Architecture and description of the biosensor

Electric cell-substrate impedance sensing has been pioneered by Giaever and Keese (Giaever and Keese, 1984) primarily to assess barrier formation and cell motility (Giaever and Keese, 1993). Besides geometries, interdigitated electrode structures (IDES) have previously been introduced to measure the impedance of biological samples (Ding et al., 2008). The sensor utilized here encased 4 sensitive elements, which can be individually addressed and thus allowing switching in case an individual element is flawed (Fig. 1A); for measurements only one element was used for data collection. Each element comprised two 50 μm wide comb-shaped electrodes which interdigitated at a gap of 50 μm over an area of 1.8 mm × 2 mm (Fig. 1B). The electrode structure was established on glass substrates, AF45 with a thickness of 0.5 mm (Schott AG, Germany) with state-of-the-art lift-off technique by first puttering a 10 nm titanium adhesion layer followed by 150 nm thick gold metallization. Sensor chips equipped with funnels molded from polydimethylsiloxan were mounted onto fixtures and then assembled with electronic tags (Fig. 1C). The wireless set-up greatly eases handling and manipulation, and also the battery-free RFID-based configuration allowed the encapsulation of measurement electronics of each tag, thereby warranting a stable operation within the humidified atmosphere of a cell culture incubator. Eventually the units were put into 6-well plastic cell culture dishes. Wireless energy and data transfer was accomplished with the aid of a radio receiver module. Thus the assembly could be placed into a cell culture incubator and was operated under sterile yet humidified conditions as described previously (Wissenwasser et al., 2010a). This re-usable system was designed to enable continuous monitoring of cell behavior and was used here for the first time to characterize cultivation and differentiation of MSC over a period of many days and weeks.

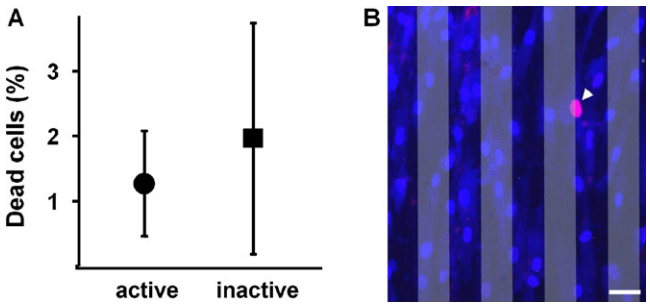

Fig. 4. Cell viability. (A) Cells were seeded on sensors and cultivated for 6 days in the presence (circle, $n = 3$) or absence (square, $n = 3$) of an electric field. Cell viability was assessed thereafter by means of fluorescent microscopy. (B) Representative epi-fluorescent micrograph of an active sensor with cultivated cells after staining with Hoechst 33342 (blue) and 7-amino-actinomycin D (bright magenta, arrow head), the latter identifying dying MSC. Approximately 800 cells from 8 non-overlapping regions per sensor were accounted, and the relative number of dying cells ± SD is depicted. The white bar indicates 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 2 shows a simplified equivalent circuit model applicable for an IDES sensor system operating at a single frequency. The model consists of the resistor R_{ser} lying in series with the capacitor C_{ser}. Cell adherence on the electrode surface results in a decrease of the effective electrode area available for electric current flow, consequently leading to an increase of R_{ser}. Whereas only a slight decline

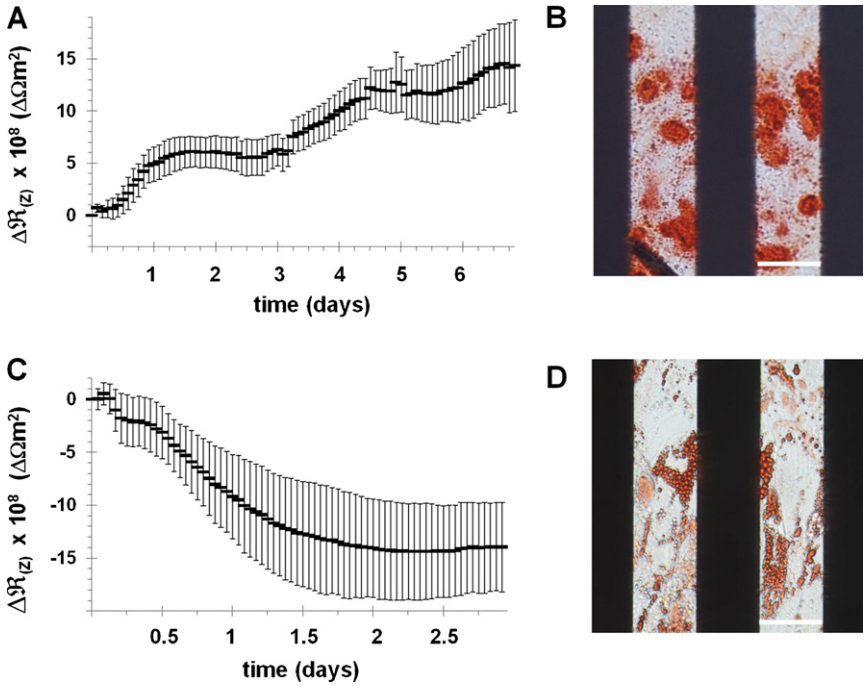

Fig. 5. On sensor osteogenic and adipogenic differentiation. (A) MSC undergoing osteoblastogenesis were monitored 7 days post induction ($n = 8$, measurement interval = 2 h). Compiled impedance signals derived from 8 parallel measurements of differentiating or untreated controls are shown. (B) On sensor osteogenic differentiation was verified by histological staining with Alizarin red S, 18 days post induction. (C) Adipogenic differentiation was assessed as described in (A) (monitoring interval = 1 h). Compiled impedance signals derived from 9 parallel measurements of differentiating or untreated controls are shown. (D) Lipid vesicles were visualized by means of Oil Red O, 12 days post induction. Mean values of differential impedance ± SD are shown. The white bars indicate 50 μm.

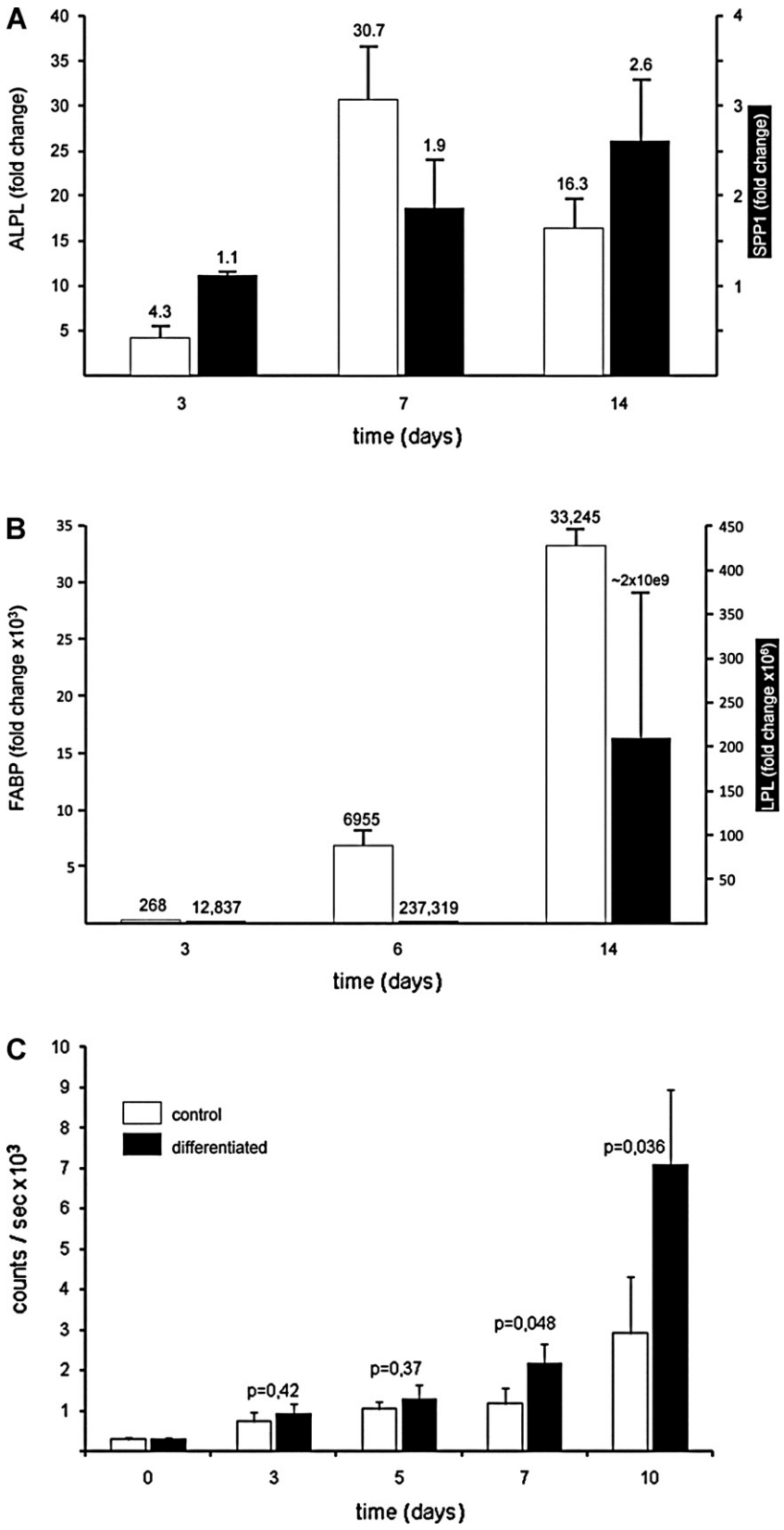

Fig. 6. Molecular marker analysis of early phases of MSC osteogenesis or adipogenesis: Transcriptional profiles were examined for (A) the osteogenic markers alkaline phosphatase, ALPL (open bars) and osteopontin, SPP1 (black bars) and for (B) the adipogenic markers fatty acid binding protein 4, FABP4 (open bars) and lipoprotein lipase, LPL (black bars). Relative transcript numbers were assessed 3, 6 and 14 days post induction by means of quantitative RT-PCR relative to the reference gene GAPDH. (C) Alkaline phosphatase activities accumulating in MSC 3, 5, 7 and 10 days post osteogenic induction were measured by means of chemiluminescence. Mean values and SD values are depicted. Results from enzymatic measurements in C were statistically analyzed with the help of an unpaired, 2-sided Student's *t*-test.

in capacity at C_{ser} was detected directly after cell seeding when cells commence with adhering to the substrate.

3.2. Cell seeding, attachment to the sensor surface and viability

Culture-expanded MSC are much sought after in regenerative medicine and tissue engineering (Salem and Thiemermann, 2010). There is a general consensus that solely quality assessment employing standardized unbiased, long-term time course measurements will warrant success in subsequent applications (Thomas et al., 2008). Moreover, reliable tools capable of measuring basic cell functionalities will not only ease the establishment of new technological approaches, but will definitely also help in optimizing currently adopted conditions and protocols in order to rejuvenate cells (Lepperdinger et al., 2008). Therefore, innovative procedures and/or new technologies, which allow continuous monitoring of MSC during *in vitro* expansion, and even more important than that, specific methods, which neither interfere with biological processes nor discretionarily interrupt cell cultivation at a defined time point for the analysis of changing biological characteristics need to be established.

To prove this concept, human bone marrow-derived MSC were seeded on a sensor, and cell adhesion was monitored by plotting the real part $\Re\{Z\}$ of the measured complex impedance over time (Fig. 3A). Immediately after applying cells a rapid increase in the ohmic resistance was observed, which then gradually decreased and eventually leveled off after approximately 24 h. At this time point, microscopic observation suggested that almost all cells had firmly attached to the sensor surface. Applied live cell imaging technique further supported this notion, since the number of loose, sphere-like cells continuously decreased while a layer of adherent, spindle-shaped MSC started to form (Fig. 3B). The interdigitated electrode fingers produce an electric field which is mainly determined by the geometrical design (Igreja and Dias, 2004) and is capable of penetrating biological cells. Compared to the culture medium, biological cells exhibit a considerably higher ohmic resistance (Irimajiri et al., 1987; Schwan, 1993). Covering the sensor surface with an excess of rounded speroidal cells alters the electric field. Hence, when cells are evenly spread and concomitantly flatten on top of the substrate, this is reflected by differential change in electric impedance. Conclusively, the time-dependent change in $\Re\{Z\}$ serves as a quantitative measure for morphological changes as a consequence of cell adhesion.

After that, we also tested the influence of the applied electric field on cell viability both during short and long-term cultivation. For this purpose MSC were seeded on active and on inactive, *i.e.* powered-off sensors. The number of dying cells was determined relative to the total cell number employed. Only a very low number of fatal casualties were detected (Fig. 4A). With respect to statistical validation of the experienced death rates, no significant difference between active and powered-off sensors was observed demonstrating that cultivation of MSC appears largely unperturbed by the applied electric field during long-term culture (Fig. 4B). Conclusively, we considered this monitoring environment amenable for any subsequent evaluation and bioassay.

3.3. Monitoring of osteogenic and adipogenic differentiation

Culture-expanded MSC are frequently used for repair of critically defected bone. As high-quality MSC readily differentiate into osteoblasts, this cell type is often considered a perfect cell source for osseous tissue engineering attempts. Aged MSC however loose this particular potential, and they are prone to undergo adipogenesis instead (Laschober et al., 2011; Moerman et al., 2004; Stolzing et al., 2008). Canonical protocols hardly allow faithful estimation of the MSCs' capacity to form osteogenic or adipogenic precursors, because the presently accepted assays strictly rely on deliberate end-point measurements.

To explore the usability of interdigitated impedance sensor for assessing the differentiation potential *in vitro*, an adherent layer of MSC was exposed to specific media either inducing osteoblastogenesis, or by stimulating the cells to turn into pre-adipocytes. When applying osteogenic stimulators, we observed steadily increasing impedance, whereas for adipogenesis a fast decrease was examined (Fig. 5A and C). For both lineages distinct changes in the impedance spectra, when compared to non-treated and undifferentiated control cells, was detectable 24 h after induction of differentiation. Later on, changes of impedance values became even more pronounced. MSC differentiation towards either lineage was further confirmed by applying specific end-point histological staining protocols (Fig. 5B and D).

In case of osteogenesis, extensive extracellular matrix production and the deposit of calcium precipitates may lead to the formation of a tight, insulating layer comprising cells embedded in a calcified extracellular matrix. It is also conceivable that the gold electrode surface absorbs ossifying extracellular matrix much stronger than the interjacent glass, and that this type of deposit alters the electrical properties of electrode structures. In any case, accumulating matrix as expected for a layer of mesenchymal progenitor cells undergoing osteogenesis, will gradually increase the measured electric resistance, an effect that has been observed previously when culturing adipose-derived stem cells on top of multielectrode arrays and measuring variations in the complex impedance Z^* (Bagnaninchi and Drummond, 2011).

Unlike to osteogenesis during adipogenic differentiation, lipid droplets are accumulating in the cytosol, which also goes along with morphological changes of the cell. Large lipid vesicle containing cells adopt a sphere-like shape, and inter-cell contacts as well as cell-substrate attachments are loosened. Conclusively, the observed decrease in resistance is likely due to facilitated current flow underneath the cells or within the gaps between rounding adipocytes.

In order to independently confirm *in vitro* adipogenic and osteogenic differentiation outcomes, expression analyses regarding specific molecular markers by means of quantitative RT-PCR together with the assessment of alkaline phosphatase enzymatic activity at early time points after induction were carried out. Results from these highly sensitive methods proved early expression of differentiation markers (Fig. 6). Notably however, sensor based validation distinguishes from RT-PCR or protein-based methods, where the cell culture is irretrievably lost.

4. Conclusions

To date standard protocols for evaluation of stem cell differentiation potential are based on end-point measurements (Scott et al., 2011; Vemuri et al., 2011). MSC differentiation is either examined with staining solutions that need to be directly applied onto cell layers followed by visual inspection, or by cell lysis with subsequent gene expression analysis. To obtain reliable and profound data, such analyses are generally conducted many days after differentiation initiation. Employing bioimpedance measurements as outlined in this contribution, distinct values can be perpetually obtained without terminating cell cultivation for good. The presented technique is distinguished for supplying profound evidence for both osteogenic and adipogenic differentiation capacity within a few days. This is crucial for rapid and reliable quality assessment in the context of medical cell production in a GMP-compliant process, needless to say that besides these specific assays, additional quality controls have to be performed at the same time which have to include immune phenotyping together with tests proving further

specific differentiation capabilities. By now, further well-defined protocols have been established for differentiating MSC *in vitro*, for instance cells exhibiting cardiomyocytic, neuronal or epithelial phenotypes (Shu et al., 2006; Park et al., 2011). As of now, it remains to be shown whether non-invasive impedance monitoring becomes also practicable in resolving characteristic properties during differentiation in a time-dependent manner. Online monitoring of cartilage formation by impedance sensing is certainly desirable yet also challenging as chondrocytic differentiation of MSC is normally accomplished in a 3-dimensional pellet culture, thus requiring a more sophisticated sensor design.

Conclusively, albeit restricted so far to cell attachment and viability as well as bipotential differentiation capacity, impedance changes as assessed by the presented interdigitated electric microsensors enables documentation of MSC quality in a fast, efficient and reliable fashion.

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