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Differentiation of mesenchymal stem cells using metal oxide thin films

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Abstract

While metal oxides synthesized as thin films have attracted much attention due to their electronic applications, we show here that they can also be used as bio-surfaces for the growth of stem cells, a field of research which has not been much explored. To demonstrate this, thin films (150–200 Å) of titanium oxide (TiO₂) and aluminum oxide (Al₂O₃) were deposited on glass using the pulsed laser deposition technique and mesenchymal stem cells were grown on these layers. Cell behavior was evaluated with respect to various key parameters, such as the composition, wettability, morphology and thickness of the films. Our results indicate that thin films of TiO₂ and Al₂O₃ can not only support stem cell adhesion and growth, but can be used to influence the osteogenic and chondrogenic differentiation paths, which opens the route for a variety of novel biocompatible materials.

Keywords: oxides, thin films, stem cells, laser ablation

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are capable of self-renewal and differentiation into multiple specific cell lineages, including muscle, fat, bone and cartilage [1]. Because of these properties, they have attracted a lot of interest in the field of regenerative medicine [2–4]. One of the challenges regarding the use of MSCs is controlling their growth either without any differentiation or, in opposition, with differentiation towards a specific cell lineage, such as chondrogenic (i.e. cartilage cells), osteogenic (i.e. bone cells) or adipogenic (i.e. fat cells) differentiation paths. MSC conversion into different cell types is the basis for cell therapy and tissue engineering and finds interest in several applications, such as orthopedic, head and neck surgery or even wound healing [5]. Therefore, it is crucial to lead cells to a certain differentiation path depending on the desired final

result, i.e. the formation of a new blood vessel, cartilage tissue, etc. To drive differentiation, many studies have been done to direct MSCs towards lineages by adding specific media. Environmental factors are also important for MSC differentiation. They include chemical factors (oxygen tension, interaction with specific extracellular components) and physical ones, such as mechanical stress, substrate stiffness, topography, etc [6–8]. Due to their unique chemical and physical properties, many studies are focused on using nanomaterials for medical applications [9–13], as they can influence the adhesion, proliferation (process involving cell division) and fate of stem cells, and the manipulation and control of cells could become possible. As an example, Nayak *et al* demonstrated that carbon nanotube thin films accelerate cell differentiation [9] and can be a suitable scaffold for tissue-engineering applications, especially bone repair. Shamsi *et al* have also shown how the incorporation of bioactive glass nanofibers in nanocomposite scaffolds can improve cell adhesion, growth and proliferation [10]. Moreover, the 3D and fibrous-porous structure of the scaffolds further contributed to their impact on cell behavior.

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Magnetic nanoparticles represent another interesting group of inorganic materials that have a close connection to living systems [14]. They offer exciting possibilities for use in the detection, manipulation and functional control of biomolecules and cells, with potential medical applications in areas, such as targeted drug delivery, magnetic fluid hyperthermia and contrast imaging. While these results on nanoparticles are interesting, these materials are not always easy to properly characterize. It is also well known that cell behavior strongly depends on the material surface properties (composition, roughness, etc) [15]. One common method of surface modification is the deposition of a thin film on a suitable substrate. As a result of these findings, metal oxide coatings have attracted attention [16–18], but this research field remains unexplored. Most studies of the potentialities of oxide thin films as scaffolds for cell growth dealt with titania surfaces, either rough or patterned. Various techniques, such as magnetron sputtering and dip coating, have thus been used for titanium dioxide deposition [19–22]. It has been shown that titania coatings not only have antimicrobial activity [23], but can enhance cell adhesion and proliferation [24, 25].

The determining factors to identify the potential of a material include its thermodynamic stability, resistance to corrosion, non-toxicity and of course the fact that it does not induce allergic reactions. Actual state of the art suggests that the surface topography is the determining parameter that affects cell behavior and differentiation [26], through the interactions with the cell surface adhesion proteins, such as integrins that modulate the cell adhesion, gene expression and overall cell behavior. At the microscale level, surface features that are of the same order of magnitude as the cell only play the role of contact guidance for the whole-cell orientation. The important structuring for the cell fate lies at the nanoscale, with elements that are of the same size range as the cell receptor. Indeed, the cells perceive their surrounding topography and their integrin receptors are triggered for adhesion by nanostructures that mimic the extracellular matrix present *in vivo* [26]. This interaction dictates the biophysical state of the cell, can modulate the cellular program or even determine cell fate. Most studies of cell growth on oxide thin films have dealt with model surfaces, prepared by standard top-down micro/nano microelectronic fabrication techniques (typical surfaces covered with pillars, with systematic variation in size, dimension and pitch). On the one hand, the drawbacks are that these techniques are expensive, difficult to scale up to large production and optimized for a limited number of materials, while on the other hand, the fact that the resulting surfaces lack irregularity seems to be beneficial to cell adhesion. Alternatively, attempts have been made to produce disordered surfaces with micro/nano topography using surface-altering techniques (polishing, etching, plating, anodizing, etc), but while cell adherence has been improved, the results are difficult to understand due to the lack of reproducibility. To improve these tissue-engineering methods, the development of new functional biomaterials is clearly necessary. Oxide thin films that are usually utilized for their electronic properties and spintronic devices [27], are another attractive option, since the control and analysis of their surfaces are simplified. In addition, deposited films can

improve corrosion resistance and prevent unfavorable developments that can occur during the release of the biomaterial's components [28, 29]. Unfortunately, (i) a very limited number of oxides has been tested so far to obtain a clear picture. Besides TiO₂, past investigations were done only on ZnO and ZrO₂ [30–32], (ii) there are only a few studies concerning the influence of metal oxide films on the behavior of human stem cells, (iii) the mechanisms of the cell-material interactions are still not completely clear and (iv) more importantly, the impact of the stem cell contact with oxide surfaces upon their differentiation has not been explored so far, which limits the development of biomedical devices. Consequently, to prepare a series of oxide films with low roughness by using a suitable method, study their biological responses and investigate their final differentiation, is the subject of the present paper. In this study, TiO₂ and Al₂O₃ thin films are prepared and deposited on glass substrates using the pulsed laser deposition (PLD) technique, a physical vapor deposition technique where a high-power pulsed laser beam is focused inside a vacuum chamber to strike a target of the material to be deposited [33, 34]. This technique is appropriate for the growth of high-quality oxide films since it is very versatile and reliable, and thus suitable for fast screening compositions. We chose the two compositions TiO₂ and Al₂O₃ because (i) TiO₂ material is often used in studies related to a common Ti-based implant and (ii) Al₂O₃ compound is investigated for other medical applications. In addition, we have a wealth of experience in their preparation and characterization. We employed human MSCs derived from bone marrow, which were directly cultured on the surfaces of the thin films and their adhesion, growth and differentiation were further evaluated. We reveal that TiO₂ and Al₂O₃ thin films can not only support MSC adhesion and growth, but, more interestingly, be used to influence the stem cell differentiation path. Therefore, our study confirms not only the potential of oxide thin films for stem cell growth, but their importance for their influence on the differentiation of stem cells, which can further be very advantageous in terms of cell-based therapies.

2 Experimental

2.1 Preparation and characterization of oxide thin films

Thin films were deposited on glass substrates using the PLD technique [35]. Briefly, this technique uses high-power laser (KrF excimer laser, with $\lambda = 248$ nm, with a laser fluence close to 2 J cm^{-2} and a laser frequency of 5 Hz) pulses to melt, evaporate and ionize the material from the surface of a target (here TiO₂ and Al₂O₃ ceramics prepared by a standard solid-state route). The ablated material is further collected on an appropriately placed substrate (glass in the present case) upon which it condenses and the thin film grows. For each sample, the number of laser pulses is fixed at 5000, leading to a total thickness of about 150–200 Å (see hereafter the x-ray reflectivity (XRR) analysis). The deposition was carried out at 300 °C. Prior to each deposition, the vacuum chamber was evacuated down with a turbo pump to a base pressure lower than $1.33 \times 10^{-4} \text{ Pa}$ (10^{-6} Torr). Samples were characterized

with XRR measurements using a Bruker D8 Discover diffractometer with $\text{CuK}\alpha_1$ radiation ($\lambda = 1.5405 \text{ \AA}$) and 2θ angle range from 0° – 6° . Thickness was fitted from the relative position of the oscillation maxima versus the square of their order number. x-ray diffraction measurements were performed using the same reflectivity setup, except for the angle range, which was from 10° – 50° . The topography was established by atomic force microscopy (AFM, PicoSPM) technique. The root-mean-square (rms) roughness (i.e. the arithmetical deviation of the mean) was derived from AFM measurements of a $1 \mu\text{m}^2$ area in the tapping mode and calculated with WSxM 5.0 software [36]. Wettability is a solid-liquid-vapor interfacial phenomenon that is characterized by measuring the water contact angle formed between a liquid drop and a solid surface. Here, the contact angle of surfaces was thus evaluated using a drop shape analysis system (DSA25B, Kruss, Germany) using the sessile drop technique ($1 \mu\text{l}$ droplet).

22 Derivation and culture of human bone marrow mesenchymal stem cells (hBMSCs)

Bone marrow was obtained from the iliac crests of adult donors undergoing arthroplasty, who had signed an agreement form, according to a local ethics committee. Marrow was obtained and cells were separated on a Hypaque–Ficoll density gradient. Mononuclear cells were taken and fixed at a density of 5×10^4 cells cm^{-2} . They were cultured in a solution of α -MEM supplemented with 10% fetal calf serum (Invitrogen, Cergy-Pontoise, France), 2 mM L-glutamine, 1 ng ml^{-1} FGF-2 (Sigma Chemical Co.) and antibiotics. When the confluency was close to 80%, the cells were harvested by trypsinization (0.25% trypsin/1 mM EDTA, Invitrogen) and seeded at 2×10^3 cells cm^{-2} . After five passages, the cells were checked for hematopoietic marker absence by real-time reverse transcription polymerase chain reaction (RT-PCR) and these hBMSCs were used for experiments. The media were changed three times per week and kept at a temperature of 37°C under an atmosphere of 5% CO_2 .

23 Cell adhesion and proliferation

hBMSCs were seeded with α -MEM medium at a density of 10^4 cells cm^{-2} in a 24-well plate onto different substrates, including thin films of TiO_2 and Al_2O_3 and uncoated glass slide as a control. After 2 h, the media were removed and the cells were washed with phosphate-buffered saline (PBS) to remove non-adherent cells. Adherent hBMSCs were then harvested by trypsinization (0.25% trypsin/1 mM EDTA, Invitrogen) and numbered separately using a trypan blue staining and Countess II Automated Cell Counter (Thermo Fisher Scientific). The same procedure was carried out to count cells after culturing at different times (2, 4 and 7 d) for proliferation monitoring.

24 Histological staining

hBMSCs were seeded at a density of 10^4 cells cm^{-2} in a 24-well plate on different substrates. The media were changed

three times per week and kept in a 5% CO_2 atmosphere at 37°C for 14 d. The results of Masson's Trichrome staining were observed with an EVOS FL Auto 2 Imaging system (Thermo Fisher Scientific).

25 Scanning electron microscopy observations

hBMSCs cultured for one and two weeks were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer ($\text{pH} = 7.4$) at 4°C , rinsed with cacodylate buffer and postfixed in 1% osmium tetroxide. Then, they were dehydrated progressively with ethanol (70%–100%) and dried in a critical point dryer (CPD 030 LEICA Microsystem). Samples were sputtered with platinum and further observed with a SUPRA 55 scanning electron microscope (Zeiss). The images were recorded at low tension (2 kV) using secondary electron (SE) and backscattered electron (BSE) detectors.

26 Differentiation assays

To investigate the differentiation activity of cells on oxide thin films, alizarin red and alcian blue staining tests were done. As a cationic dye, alcian blue binds strongly to sulfated glycosaminoglycans and glycoproteins, while alizarin red, an anionic dye, binds to cationic metals, such as calcium [37]. Note that MSCs were cultured with standard α -MEM medium (without basic fibroblast growth factor). After 7 d, the osteogenic and chondrogenic media were added for alizarin red and alcian blue staining, respectively. The medium was changed each 2–3 d in both cases. After 14 d of culture in total, the cells were fixed with 4% paraformaldehyde (PFA) and after being washed with PBS, were kept in 1.5 ml of PBS for subsequent differentiation staining tests. In brief, after aspiration of PFA, the cells were washed with distilled water (H_2O) and stained with alcian blue or alizarin red solution for 30 min. After alizarin red staining, the cell samples were washed four times with H_2O and fixed on microscopic histological glass slides with an acrylic polymer solution. For optical density analysis of alcian blue staining, the cell samples were washed with H_2O and after 1% sodium dodecyl sulfate was added and subsequent lysates, were transferred to a 48-well plate. The absorbance at 616 nm was determined for triplicate samples (Multiskan GO spectrophotometer, Thermo Scientific).

27 Real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR technique was used to measure the expression levels of genes related to the osteogenic and chondrogenic differentiation of MSCs. Depending on their involvement in the particular type of differentiation process, the gene markers were chosen accordingly: OMD, RUNX2 and SPARC genes were tested after cell culture in osteogenic medium, COL2, ACAN, COL1, COMP and SOX9 were tested after cell culture in chondrogenic medium. The sequences of these primers can be seen in table 1.

The cell culture was done using standard α -MEM medium (without basic fibroblast growth factor). The medium was

Table 1. Primer sequences used for PCR analysis.

Gene	Primer
RUNX2 (runt-related transcription factor 2)	F: GTGATAAATTCAGAAGGGAGG R: CTTTTGCTAATGCTTCGTGT
OMD (osteonmodulin)	F: AGGCTGTGTCAGTGAATGCTT R: TTGGATAGTCTTGAGTTTGCAT
SPARC (secreted protein acidic and cysteine-rich)	F: CCCATTGGCGAGTTTGAGAAG R: CAAGGCCCGATGTAGTCCA
COL1 (collagen type I alpha 1)	F: CACCAATCACCTGCGTACAGAAC R: CAGATCACGTCATCGCACAAAC
COL2 (collagen type II)	F: TGGACGATCAGGCCGAAACC R: GCTGCGGATGCTCTCAATCT
ACAN (aggrecan)	F: GTGCCTATCAGGACAAGGTCT R: GATGCCTTTCCACCACGACTTC
COMP (cartilage oligomeric matrix protein)	F: GCTCAGGGCCCTCATAGAT R: TGGGTTGGAAGGACAAGAAG
SOX9 (SRY (sex determining region Y)—Box 9)	F: CCCATGTGGAAGGCAGATG R: TTCTGAGAGGCACAGGTGACA
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	F: ATGGGGAAGGTGAAGGTCTG R: TAAAAGCAGCCCTGGTGACC
RPL13 (ribosomal protein L13)	F: GTTCGGTACCACGAAGGT R: CTGGGGAAGAGGATGAGTTTG
B2MG (beta-2-microglobulin)	F: GAGGCTATCCAGCGTACTCCA R: CGGCAGGCATACTCATCTTTT
Actin (beta-actin)	F: AGACCTGTACGCCAACACAG R: CGATCCACACGGAGTACTTG

refreshed twice per week. Total RNA, after 14 d of cell culture, was extracted using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Five hundred ng of the extracted RNA were then DNase I treated and reverse transcribed into cDNA (Invitrogen kit). GAPDH, RPL13, B2MG and Actin housekeeping genes were used to normalize the mRNA expression levels calculated by the $2^{-\Delta\Delta Ct}$ method.

3. Results and discussion

3.1 Film characterization

Figure 1(a) displays typical AFM images of TiO₂ and Al₂O₃ films taken at room temperature and atmospheric pressure. Both compositions have a uniform nanostructured surface with rms values in the range of 0.1–0.5 nm, indicating that splashing is not significant. Note that these values are significantly lower (by 2–3 orders of magnitude) than those usually obtained with commonly used magnetron sputtering or chemical vapor deposition methods [20, 38]. This confirms that PLD is a fast, efficient, reproducible and most appropriate technique for producing smooth and well-controlled oxide films [35, 39]. We also note that the rms values are very similar between the film series (among more than 100 of the samples tested) and that all films have grain size from 90–110 nm, leading to an average of less than 100 nm. The range of film roughness values was independent of the growth conditions, and thus, the surface topography variations can be neglected. Since all the films have a very smooth and flat surface, which does not impose any additional stress on the cells, we further consider only the

influence of the composition of the films on the results, as discussed below.

Surface wettability properties were also investigated by means of measuring water contact angles (figure 1(a), top right). The contact (wetting) angles were calculated to be 72.02° and 85.30° for TiO₂ and Al₂O₃, respectively. This suggests that the aluminum oxide surface is more hydrophobic than the titanium oxide one. There was no significant difference in the contact angles observed between the various growth conditions (temperature, oxygen pressure), which is in agreement with previous reports done on coated silicon showing that the gas pressure did not affect the contact angles [40]. While Wenzel equation states the relationship between surface roughness and wettability, we did not observe a significant effect. Several films were indeed prepared and the roughness was always within the same range [42]. Overall, the analyzed surfaces are hydrophilic, with a water contact angle of less than 90 degrees. After this high number of screening experiments, we decided to only consider the chemical composition as the main parameter of our study.

To evaluate the structural quality, θ - 2θ x-ray diffraction analysis was performed. The resulting diffractograms displayed flat broad peaks with very low intensity (close to the background), confirming that all films are amorphous. This amorphous nature is attributed to the low-surface mobility of particles (due to a low deposition temperature, below 350 °C) and the utilization of glass substrate (which is also amorphous). XRR experiments were also performed (see figure 1 for a typical pattern). Data were analyzed with fitting procedures to determine the film thickness from the period of oscillation curves, which appear as a result of interference

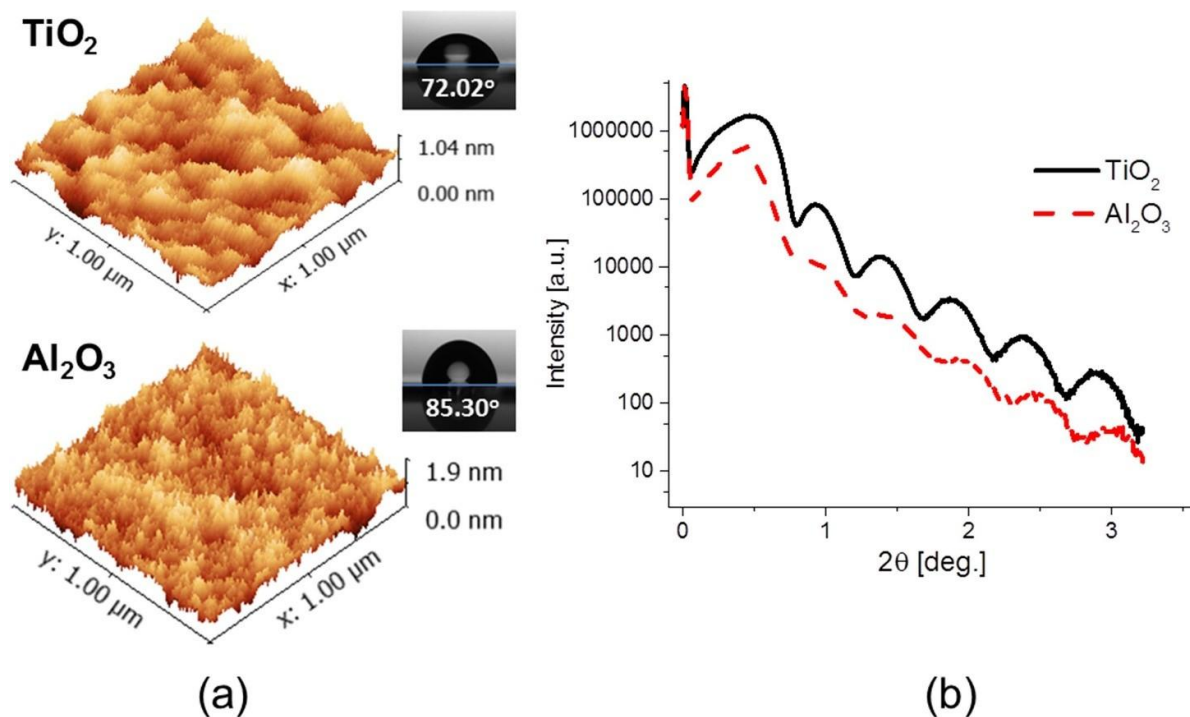


Figure 1. Characterization of oxide films prepared on glass substrates using the PLD technique. (a) 3D AFM images of TiO_2 (top) and Al_2O_3 (bottom) films. Films are continuous, with an average surface roughness lower than 1 nm (extracted from the software) [36]. Water contact angle measurements are presented on the right side of the AFM images. Lower value of the angle between both films indicates that the TiO_2 surface is more hydrophilic than the Al_2O_3 one (72.02° versus 85.03°). (b) XRR curves of TiO_2 (solid line) and Al_2O_3 (dash line) films. Thickness of films was extracted from the position of oscillation maxima and found to be in the range of 15–20 nm. See text for details.

between the x-rays scattered from the surface of the thin film and the interface between the substrate and thin film. From the XRR analysis, the thickness of the deposited films was found within the range of 15–20 nm. Taking into account the number of laser pulses during the growth (fixed at 5000), the deposition rate (i.e. the thickness of the layer material deposited per pulse) is calculated to be equal to 0.03–0.04 Å/pulse ($= (150\text{--}200 \text{ \AA})/5000$ laser pulses), confirming very precise control of the thickness, which is another advantage of the PLD technique [35].

3.2 Adhesion and proliferation of hBMMSCs on TiO_2 and Al_2O_3 thin films

hBMMSCs were grown on three different classes of surfaces: (1) TiO_2 thin films deposited on glass, (2) Al_2O_3 thin films deposited on glass and (3) unprocessed glass cover slip (on top of which no coating was deposited) that served as the control. Two hours after seeding, the hBMMSCs displayed a rounded shape (not shown). The calculation of the number of adherent cells indicates that there is no significant difference between substrates coated with oxide thin films and the glass control (see figure 2(a)). With respect to the cell proliferation process (which includes both cell division and growth), we also observed that the number of cells after 2 and 4 d is similar for all studied samples, indicating that neither oxide interferes with normal division in the early stages of cell culture (figure 2(b)). After a few days, the situation is slightly

different. The higher number of cells on the glass control and TiO_2 after 7 d of culture is associated with their lower contact angle value, which facilitates the cell adhesion at least in the initial step of the growth [41]. It is worth noting that although there are a number of studies showing how the higher wettability is beneficial for the cell proliferation [43, 44], it was also demonstrated that in some cases cells preferred to attach and grow on the surface with a suitable combination of roughness and wettability, i.e. with neither the highest nor the lowest surface energy values [19]. These preliminary results show that the cell growth is influenced by the surface properties including its composition and wettability, although these effects can also depend on the duration of the culture. Considering the initial density of adherent cells for each condition, these results illustrate that the parameters such as the surface hydrophilicity are also important with respect to the cell development and growth. The effect of this parameter on cellular response is actually related to its influence on the protein–surface interaction, which is known to play a vital role in many processes regulating cell behavior [45, 46].

After the initial stage of adhesion, MSCs started to proliferate and within the first days of culture they displayed an elongated shape without any preferential orientation. To highlight this cell morphology in the later stages of culture, Masson’s trichrome staining was used (see figure 2(c)). Using a three-dye-based protocol, this staining allows one to observe not only cell shape, but (in case they are present) selectively visualize components related to the differentiated state of the cell. The MSCs

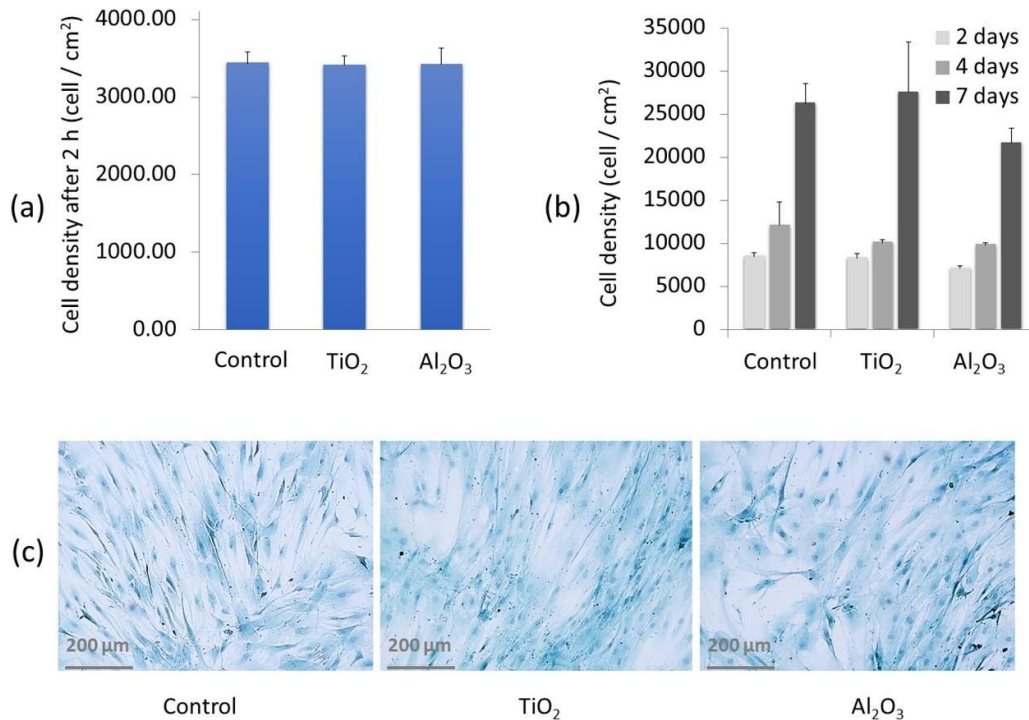


Figure 2. (a) Cell density after 2 h of culture. (b) Number of MSCs on TiO₂ and Al₂O₃-coated substrates, as well as on the uncoated glass substrate. Corresponding number measured after 2, 4 and 7 d of culture. These results show that oxide thin films do not interfere with normal proliferation in the early stages of cell culture. (c) Phase contrast optical microscopy images of trichrome Masson staining after 14 d of cell culture on the glass control, TiO₂- and Al₂O₃-coated substrates.

cultured for 14 d on the three types of surfaces (both thin films, as well as on the glass control), exhibited spindle-like shape, which is typical for this cell type. Maintaining the morphology associated with the undifferentiated state of MSCs can be very important in terms of their differentiation potential, as was demonstrated in some studies that showed how a differentiation path of a stem cell can be directed by certain changes in morphology [47–49]. In addition, uniform cell organization was noted with no significant inhomogeneity in cell spreading between the different samples, indicating healthy morphology of the cultured MSCs.

The evolution of cell growth and spreading was further analyzed by means of scanning electron microscopy (figure 3). After 7 d of culture, flattened MSCs with sizes within the range of 10–20 μm can be distinguished on the glass control, as well as on the TiO₂ and Al₂O₃ thin films (figures 3(a)–(c)). After 14 d of cell culture, the BSE images (figures 3(d)–(f)) allowed us to obtain a better view of the extracellular matrix. Deposition of the matrix was observed for all conditions with similar cell organization, as it can be seen that cells on both oxides and the control glass adopt a linear alignment with good coverage of extracellular matrix.

33 Influence of TiO₂ and Al₂O₃ coatings on osteogenic and chondrogenic differentiation of hBMMSCs

The impact of TiO₂ and Al₂O₃ thin films upon the MSC differentiation was done by various staining assays. These assays use specific chemical reactions to identify a particular differentiation path. It is worth noting that the osteogenic

potential of hBMMSCs was first investigated due to the faster cell growth for this condition. Cells were cultured in osteogenic medium and, in order to mark calcium-rich deposits associated with osteoblasts (bone cells), alizarin red staining was used. Although these calcium-rich sites of mineralization were not detected in large quantities by optical microscopy observations, the staining of cells that were grown on Al₂O₃ appears to be more pronounced than that on TiO₂ or the glass control (figure 4(a)). With regard to the other features of osteogenesis, the change to polygonal shape, which is typical for cells undergoing differentiation to osteoblasts, could be partially seen for cells cultured on Al₂O₃. Overall, the lack of pronounced signs of osteogenesis can be explained by the reduced duration of culture with differentiation medium. Indeed, it was reported that the transformation of MSCs into osteoblasts can occur with cell cultures for 21 d [50]. In our case, the period of culture was reduced due to a limitation associated with the highly confluent state reached after two weeks.

The influence of oxides upon chondrogenic differentiation, which is related to the formation of cartilage cells (chondrocytes), was done in a second step. After culturing in chondrogenic medium, MSCs were used for alcian blue staining. The quantification of this staining by spectrophotometry is presented in figure 4(b). Taking into account standard error, one can observe the effect of both oxide thin films on the initiation of chondrogenesis. In particular, Al₂O₃ showed higher values for the absorbance of eluted alcian blue, suggesting a positive influence of this aluminum oxide on the chondrogenic differentiation of MSCs.

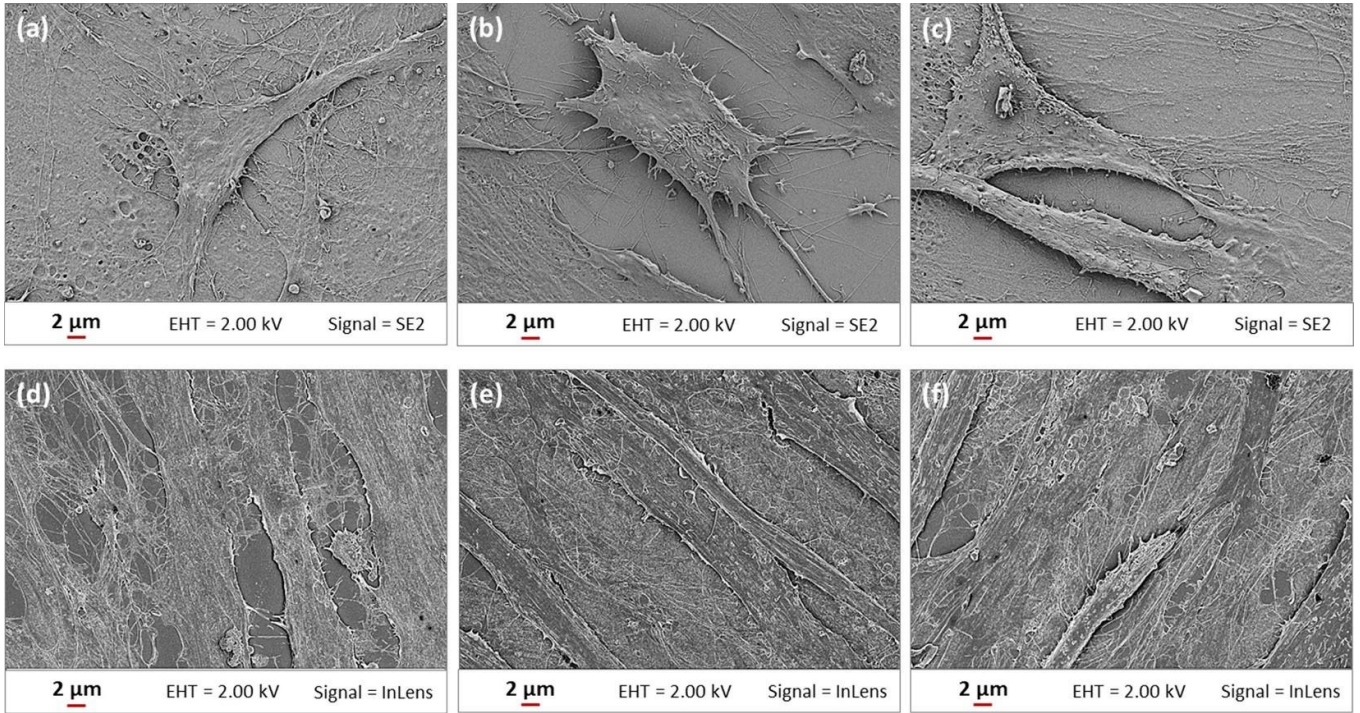


Figure 3. SE and BSE SEM images recorded after 7 d (top) and 14 d (bottom) of cell culture on the glass control, respectively. (a), (d) glass control, (b), (e) TiO₂-coated substrates, (c), (f) Al₂O₃-coated substrates.

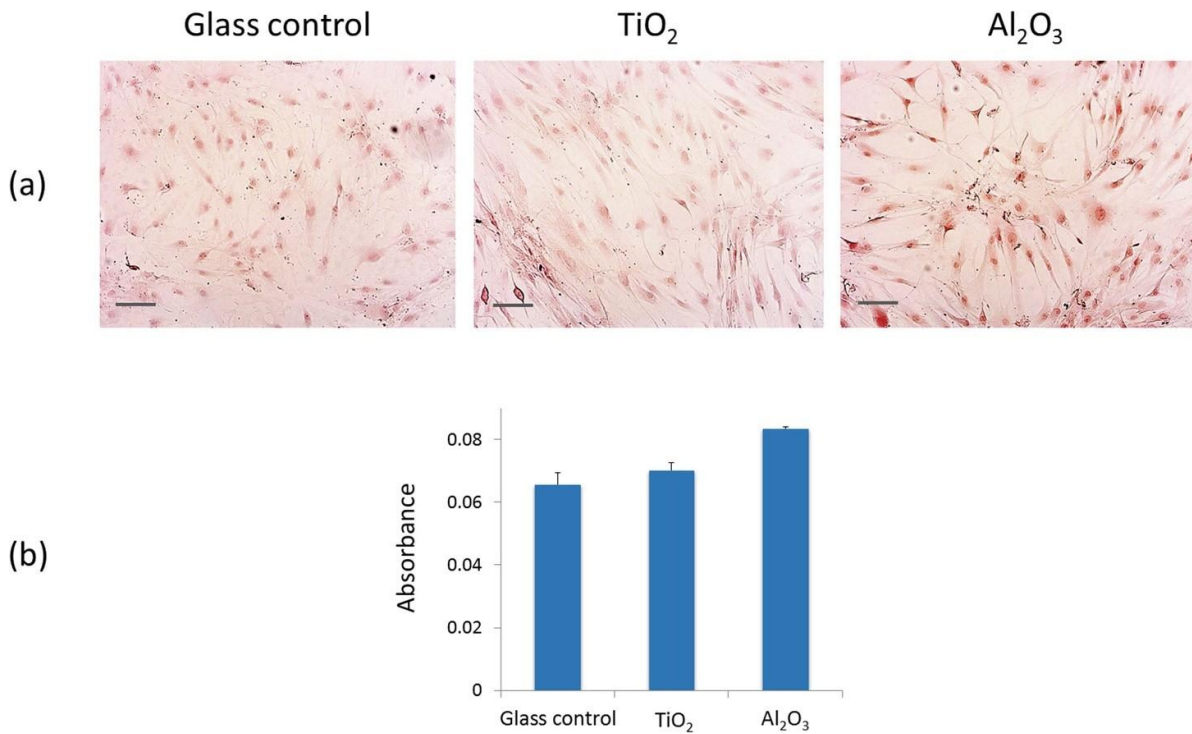


Figure 4. (a) Alizarin red staining of MSCs seeded on the glass control, TiO₂- and Al₂O₃-coated substrates after 14 d of cell culture; scale bar = 100 μm. No significant difference for osteogenic differentiation is observed. (b) Absorbance of the eluted alcian blue for MSCs seeded on the glass control, TiO₂- and Al₂O₃-coated substrates after 14 d of cell culture. Taking into account standard error, results indicate the positive influence of Al₂O₃ on the chondrogenic differentiation of MSCs.

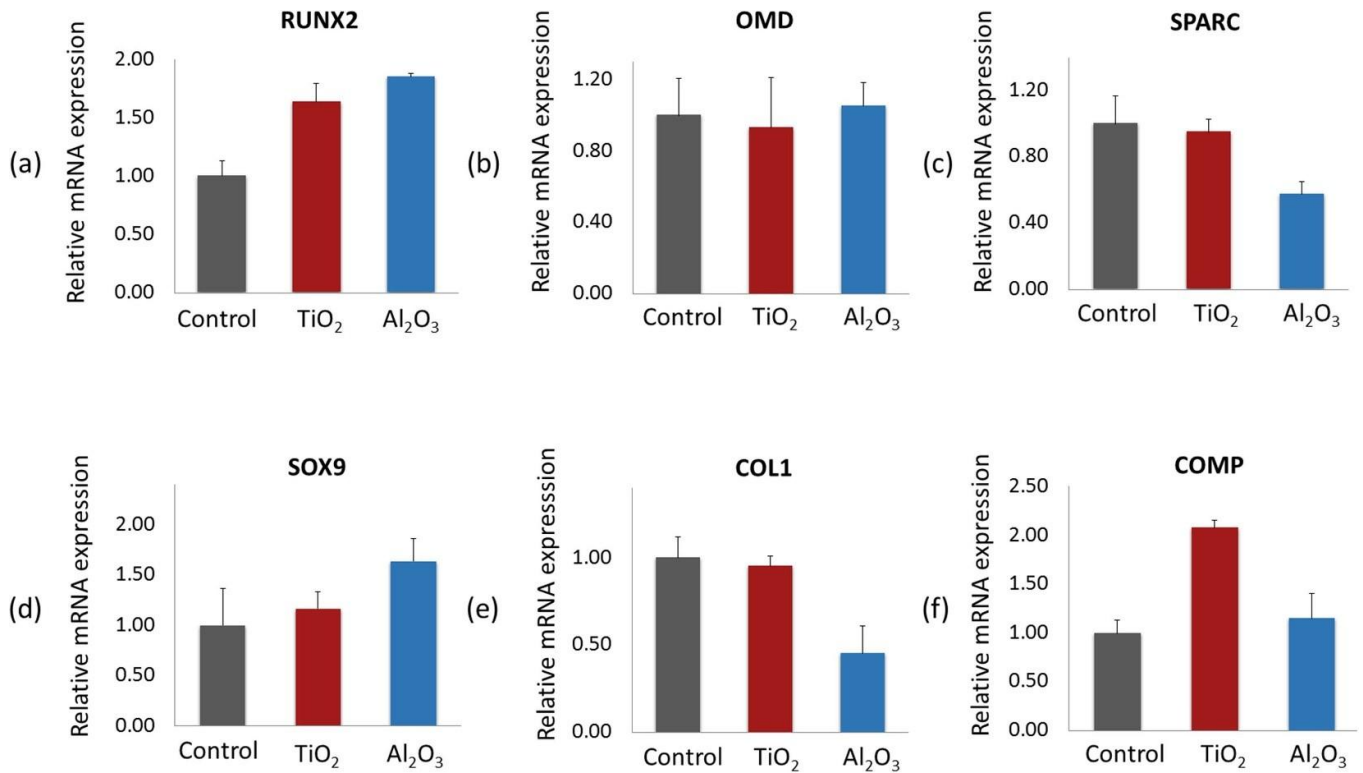


Figure 5. Osteogenic and chondrogenic differentiation of hBMSCs on the various samples after culturing for 14 d in standard α -MEM medium. Real-time RT-PCR analysis of osteogenesis-related genes, (a) RUNX2, (b) OMD, (c) SPARC and chondrogenesis-related genes; (d) SOX9, (e) COL1, (f) COMP (see text for details). Statistical evaluation is performed by comparing the samples with the glass substrate.

This result is interesting because the effect of Al₂O₃ on MSC chondrogenic differentiation has not been previously reported, while very few studies concerning the effect of TiO₂ (nanotubes but not thin films) on chondrogenic differentiation of MSCs can be found [51, 52].

To evaluate the expression levels of genes, which illustrate the disposition of stem cells towards specific differentiation, real-time RT-PCR analysis was performed. Typical OMD, RUNX2 and SPARC genes were tested for osteogenesis (i.e. bone formation), while COL2, ACAN, COMP and SOX9 genes were tested for chondrogenesis (i.e. cartilage formation). In order to obtain complementary data about MSC chondrogenic differentiation, expression of the COL1 gene that encodes one of the most abundant proteins was analyzed as well. After cell culture for 14 d in standard α -MEM media without bFGF, a difference in gene expression was noted.

In order to assess the influence of oxide thin films on the osteogenic differentiation of MSCs, we analyzed data on osteogenic markers (figures 5(a)–(c)). It can be seen that more RUNX2 was expressed in cells seeded on TiO₂ and Al₂O₃ thin films compared to the glass control (figure 5(a)). No significant difference in expression levels between the glass control and oxide thin films was noticed for another important osteogenesis-related gene, namely osteomodulin (OMD), which is related to the biomineralization process. In the case of SPARC expression, its down-regulation can be observed for cells seeded on Al₂O₃ (figure 5(c)). The difference between the effect of Al₂O₃ coating on the expression of RUNX2

and SPARC can be explained by their different role in the osteogenesis. While SPARC (also known as osteonectin) is involved in the process of calcium ion binding and bone mineralization, RUNX2 is the transcription factor that has a primary role in early osteogenic differentiation and is considered the key marker of osteogenesis. Therefore, our data on the expression of RUNX2, along with the results of differentiation staining assays, indicate a positive influence of both oxide thin films on the osteogenic differentiation of MSCs, which has not been reported to date.

Moreover, the results on the effect of TiO₂ and Al₂O₃ thin films on the osteogenic differentiation of MSCs obtained within this study are actually consistent with the literature data previously reported. Although there have been few reports on the effect of TiO₂ and Al₂O₃ materials on directing MSCs towards osteogenesis [53–57], only one of them deals with thin films. To be more precise, Weng *et al* employed a thick double-layered Al₂O₃ coating with a porous outer layer to showcase its potential to promote bone growth both *in vitro* and *in vivo* [56]. However, in this case, the improvement in osteogenic activity is likely promoted by the porosity of the coating surface along with the presence of doping elements, while in our study, since smooth non-porous thin films were used, we assess the effect of different surface chemistry on osteogenic differentiation.

Concerning the chondrogenic differentiation of MSCs, altered levels of COL1 and some chondrogenic markers, such as COMP and SOX9, can be observed in figures 5(d) and (f). From the data that were obtained on chondrogenic

transcription factor SOX9, it can be seen that in comparison to other samples, Al₂O₃ enhances the SOX9 expression after cell culture in standard medium (figure 5(d)). As SOX9 is greatly involved in the regulation of chondrogenesis, its elevated levels for cells seeded on Al₂O₃ demonstrate the potential of using this oxide film to increase the disposition of MSCs towards chondrogenic differentiation. Interestingly, significant down-regulation in the case of Al₂O₃ can also be observed for the expression of COL1 (figure 5(e)). The expression of this gene is substantially related to fibrocartilage, the formation of which is undesired during cartilage defect restoration therapies [58]. Therefore, our data are interesting for driving stem cells to differentiate into particular types of cartilage that are used in tissue engineering. It is noteworthy that the expression of ACAN and COL2 genes, which are the primary markers of chondrogenesis, was detected only partly during these tests. This indicates the early stage of chondrogenesis, as these genes are expected to be seen at a later stage. Despite the fact that the expression of ACAN could not be observed properly, the altered levels for another gene related to the non-collagenous matrix, namely COMP, were detected. Elevated levels of this gene expression can be noticed, in particular, for cells seeded on TiO₂ thin films with relative mRNA expression twice as high in comparison to the control sample. A similar effect of TiO₂ on the chondrogenic differentiation of MSCs has already been reported [51]. However, Park *et al* studied TiO₂ in the form of nanotubes, while the present study is focused on using oxide thin films. Moreover, to the best of our knowledge, there have been no studies on the potential impact of Al₂O₃ and, in particular, in the form of thin coatings on the chondrogenic differentiation of hBMSCs. In summary, the results of differentiation staining assays and real-time RT-PCR analysis are in good accordance, both indicating the potential of using oxide thin films to influence MSC fate.

Further studies would be valuable to see how oxide thin films affect the cell differentiation path in the case of long-term cell cultures and are already in progress. Complementary information can be obtained by the evaluation of the ability of oxide thin film to bind to specific proteins provided by the culture media. For example, compared to graphene, the higher adsorption capacity of graphene oxide for serum proteins resulted in a higher density of adhesion molecules available for cell attachment and growth [59]. Furthermore, it was reported that the osteogenic differentiation of hBMSCs was enhanced by the capacity of graphene to fix β -glycerophosphate and dexamethasone, while the adipogenic specialization was improved by the ability of graphene oxide to fix insulin. Moreover, both the acceleration and inhibition of hBMSC growth can also be explained by a potential difference related to the protein adsorption ability of oxide thin films.

4. Conclusion

Human bone MSCs were grown on three different surfaces, namely, smooth amorphous thin films (150–200 Å) of

titanium oxide (TiO₂) and aluminum oxide (Al₂O₃), deposited on glass substrates using the PLD technique, and a glass control. The observations of cell proliferation indicated that hBMSC culture onto films results in a normal cell pattern. No significant difference in cell appearance was observed when hBMSCs were cultured on both thin films and the control, but an acceleration related to the cell growth was, however, observed in the case of TiO₂ thin films. Cell differentiation was also explored and we found that osteogenic and chondrogenic differentiations are influenced by the nature of the surface chemistry, i.e. TiO₂ and Al₂O₃ thin films. In addition to what has been previously reported, we demonstrate that even smooth non-porous TiO₂ and Al₂O₃ thin films can not only support the growth of hBMSCs, but affect their osteogenic differentiation. Furthermore, we note that there have been no studies on the potential impact of Al₂O₃, in particular, in the form of thin coatings on the chondrogenic differentiation of hBMSCs. Our results indicate that Al₂O₃ coating appears to be superior for providing the enhancement of the SOX9 gene expression related to the chondrogenic differentiation of MSCs. This observation is consistent with the results of alcian blue quantification, where higher absorption was observed for Al₂O₃ samples. Interestingly, a significant decrease in the COL1 expression was noted for Al₂O₃ thin films. Thus, it was demonstrated how Al₂O₃ thin film can be preferential from the perspective of its influence on the chondrogenic differentiation of MSCs with simultaneous suppression of the COL1 expression, which can be advantageous in cell therapies. Further studies should be carried out to determine the precise link between the protein adsorption profile of oxide thin-film surfaces, cell proliferation and impact of oxide thin films on MSC differentiation.

From a broader perspective, these results clearly demonstrate that oxide thin films can be used to influence cellular behavior and differentiation. Therefore, metal oxide thin films are considered as a promising platform for stem cell culture and the study of cell behavior, thus expanding the range of their applications beyond oxide electronics.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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Author contributions

W P and K B conceived the experiments. M K, M H and E L performed the sample fabrication, cell growth and data analysis. M K, R R and D G performed the microscopy experiments. A F, A D and U L helped with the analyses. All the authors contributed to the manuscript. W P and K B supervised and guided the project.

Methods and protocols

All methods were carried out in accordance with relevant guidelines and regulations and all experimental protocols were approved by the 'Comite de la Recherche en Matiere Biomedicale et de Sante Publique'. Informed consent was obtained from all the donor participants.

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