Investigation of early cell–surface interactions of human mesenchymal stem cells on nanopatterned β-type titanium–niobium alloy surfaces

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Multi-potent adult mesenchymal stem cells (MSCs) derived from bone marrow have therapeutic potential for bone diseases and regenerative medicine. However, an intrinsic heterogeneity in their phenotype, which in turn results in various differentiation potentials, makes it difficult to predict the response of these cells. The aim of this study is to investigate initial cell–surface interactions of human MSCs on modified titanium alloys. Gold nanoparticles deposited on β-type Ti–40Nb alloys by block copolymer micelle nanolithography served as nanotopographical cues as well as specific binding sites for the immobilization of thiolated peptides present in several extracellular matrix proteins. MSC heterogeneity persists on polished and nanopatterned Ti–40Nb samples. However, cell heterogeneity and donor variability decreased upon functionalization of the gold nanoparticles with cyclic RGD peptides. In particular, the number of large cells significantly decreased after 24 h owing to the arrangement of cell anchorage sites, rather than peptide specificity. However, the size and number of integrin-mediated adhesion clusters increased in the presence of the integrin-binding peptide (cRGDfK) compared with the control peptide (cRADfK). These results suggest that the use of integrin ligands in defined patterns could improve MSC-material interactions, not only by regulating cell adhesion locally, but also by reducing population heterogeneity.

1. Introduction

Owing to the steadily increasing number of elderly people and the resulting rise in age-related diseases, such as osteoporosis and osteoarthritis, there is a high demand for long-lasting and biocompatible orthopaedic implant materials [1]. Nowadays, titanium (Ti) alloys are widely used for orthopaedic and dental implants owing to their mechanical characteristics, biocorrosion resistance and adequate biocompatibility. However, a significant discrepancy between the high elastic moduli (more than 100 GPa) of materials currently used for orthopaedic implants, such as cp-Ti and Ti–6Al–4V, and the low elastic modulus (less than 30 GPa) of bone, impairs bone turnover at the implant site. This eventually leads to implant failure, also called the stress-shielding effect [2]. Moreover, depending on the alloying element, biocompatibility issues need to be carefully considered, especially for in vivo situations. Ti–6Al–4V (α + β type), the most commonly
used Ti alloy, was reported to trigger immunologic reactions in hip replacements [1,3]. Dissolved vanadium and aluminium were shown to induce severe reactions within the tissue and to affect growth rates in fibroblasts and osteoblasts [2,4,5].

Therefore, more biocompatible β-type Ti alloys with minimal side effects and satisfactory mechanical features have been developed. These alloys contain β-stabilizing elements, such as niobium (Nb), tantalum and zirconium, and exhibit superior mechanical properties, e.g. much lower elastic moduli compared with cp-Ti (α-type) and Ti–6Al–4V, as well as low metal release rates. Furthermore, these β-type alloys show excellent performance regarding the inflammatory response and osteoconductivity [6]. As for Ti–Nb alloys, the use of a high Nb content lowers the elastic moduli further, thereby rendering those alloys preferred materials for medical applications [7]. With 40–45 wt% Nb, it is possible to obtain an elastic modulus of 60–62 GPa, which can be even further lowered to 40–50 GPa by thermo-mechanical processing and microalloying [8,9].

Lately, there is an ever-growing interest in using human adult mesenchymal stem cells (MSCs) for regenerative medicine approaches. Derived from bone marrow, these cells can differentiate into a variety of lineages, including osteoblasts, chondrocytes and adipocytes [4,5,10]. Stem cell fate is also determined by their interaction with the microenvironment, namely the extracellular matrix (ECM). Stem cells are responsive to physical features of the extracellular environment, such as topography and stiffness, as well as to chemical features, such as molecular composition of the ECM and ligand density [6,11–13]. A population of MSCs from the same individual comprise a heterogeneous mixture of cells with differing differentiation and proliferation potentials [14,15]. This heterogeneity is further increased upon isolation and during in vitro selection, resulting in cells exhibiting various degrees of maturation [16,17]. In vitro senescence of MSCs is accompanied by an increase in cell size. These large-sized cells ultimately stop proliferation but can be maintained in this state for several months in culture [16].

The presence of non-proliferative senescent cells is a problematic issue in regenerative medicine. On the one hand, they may prevent adhesion and settling of desired proliferating cells, simply by covering large fractions of the implant’s surface. On the other hand, senescent cells can modify their microenvironment by inducing senescence in neighbouring and remote cells extrinsically through their altered secretome [18]. Therefore, it is of utmost significance to carefully control initial cell settling on implant materials. A general approach is the modification of the material’s surface. Thus, enhancing or preventing adhesion of these cells can be achieved by immobilization of specific ligands, such as proteins or bioactive peptides derived from the ECM [19,20].

A number of studies describe different approaches for immobilizing peptides on surfaces using self-assembled polymers. As was shown by Zorn et al. [21,22], a self-assembled monolayer was grafted to electropolished and oxidized Ti–45Nb surfaces and bioactive peptides immobilized onto this monolayer. Frith et al. [23] investigated the effect of lateral peptide spacing on the ability of MSCs to establish mature focal adhesion (FA). As already extensively shown in the past, cell adhesion to different substrates via integrins can be improved by presentation of short integrin-binding motifs found in a variety of ECM proteins. Among them, the commonly used RGD (arginine–glycine–aspartic acid) motif present in e.g. fibronectin, vitronectin and osteopontin is widely used to study MSC proliferation and differentiation [24–26]. Furthermore, RGD and the laminin-derived binding motif IKVAV (isoleucine–lysine–valine–alanine–valine), were also shown to influence, respectively, long-term viability and osteoblastic differentiation of MSCs [27]. RGD in combination with growth factors was immobilized on microspheres to construct scaffolds for MSC adhesion, proliferation and differentiation [28]. Early adhesion and spreading of primary osteoblasts on trimmed and sandblasted Ti alloys (Ti–6Al–4V) was positively affected by cyclic RGD coating, as was shown by Mas-Moruno et al. [29]. An elegant method to control the spatial density of bioactive peptides and the orientation of immobilized ligands is a dip-coating technique based on self-assembly of diblock copolymer micelles (block copolymer micelle nanolithography, BCMN) [30]. Using gold as the micelle core, a hexagonally ordered pattern of gold nanoparticles can be created on various materials, including orthopaedically relevant surfaces, for example Ti, and used to direct cell adhesion [31,32].

In this study, we present the patterning of Ti–40Nb alloy discs and showed the effects of nanotopography and biofunctionalization with integrin ligands on human MSCs early adhesion and phenotype selection. Here, we use BCMN to create gold nanopatterns on Ti–40Nb alloys. Specific functionalization of the gold nanoparticles was achieved by using the thiolated ligands cRGDfK and cRADfK. Based on the characterization of early cell–surface interactions on the modified and functionalized alloys, we demonstrate that the heterogeneity of human MSC is reduced and their adhesion, in terms of cell size and FA formation, is optimized by using the cRGDfK peptide.

2. Material and methods

2.1. Preparation of titanium alloys

High-purity Ti and Nb were arc-melted in argon atmosphere to obtain Ti–40Nb (wt%) ingots. These pre-alloys were subsequently melted by induction heating and cold crucible cast with a pressure of 500 mbar in a water-cooled copper mould with a diameter of 10 mm and a length of 100 mm. The samples were sealed in quartz tubes and homogenized in purified argon atmosphere at 1000 °C for 24 h, and subsequently water quenched. Discs of 2–3 mm thickness were cut from the rods. Finally, the alloy composition was determined by inductively coupled plasma optical emission spectroscopy (iCAP 6500 Duo, Thermo Fisher Scientific GmbH, Germany). The structural state of the cast and homogenized samples was checked with X-ray diffraction (STOE Stadi P instrument, STOE & CIE GmbH, Germany) and scanning electron microscopy (SEM, LEO 1530 Gemini, Carl Zeiss, Germany) and verified a polycrystalline single b-phase state with a mean grain size of approximately 70 μm [9].

In a standard procedure, the aforementioned 10 mm Ti–40Nb discs were plane ground with SiC emery paper up to grid P400, followed by lapping with diamond suspension (9, 6 and 3 μm) using a flat grinding plate (Struers, Germany). The final polishing was carried out with a mixture of colloidal silica and hydrogen peroxide. After the fine polishing procedure ending with a mixture of colloidal silica and hydrogen peroxide, the alloy surface was extremely smooth (mirror-like). The surface roughness was analysed with optical profilometry using a MicroProf system (FRT, Germany), whereby a surface area of 2 × 2 mm was mapped. Typical roughness data are $Ra = 0.02 ± 0.01 \mu m$. 

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were washed with sterile phosphate buffered saline (PBS). Room temperature for at least 4 h. Prior to cell plating, the discs were passivated by incubation with 0.1 mg ml⁻¹ PLL-g-PEG in HEPES (Surface Solutions, Switzerland) for 30 min at room tem- perature. Following several rinses with distilled water, the discs were incubated with 25 μM cyclic RGDfK or cyclic RADfK at room temperature for at least 4 h. Prior to cell plating, the discs were washed with sterile phosphate buffered saline (PBS).

2.2. Preparation of nanopatterns on Ti–40Nb discs
The technique of gold deposition on surfaces by BCMN is based on Glass et al. [30]. Here, we achieved efficient surface patterning by adjusting plasma treatment and surface preparation prior to the dip-coating process. Polished and ground Ti–40Nb discs were activated in oxygen plasma (0.4 mbar, 150 W, 10 min) prior to BCMN. The micelle solution was prepared in toluene with either polymer ‘1056’ (consisting of 1056 polystyrene and 571 vinyl pyridine units, 7 mg ml⁻¹) or with polymer ‘2074’ (consisting of 2074 polystyrene and 571 vinyl pyridine units, 2 mg ml⁻¹) and HAuCl₄ to obtain a gold loading of 0.5 (HAuCl₄ from Sigma, Germany). After dip-coating the discs, the organic compounds on the surface were removed by oxygen plasma (0.4 mbar, 150 W, 60 min), resulting in coalescence of the gold nanoparticles. Passivation and biofunctionalization of nanopatterned (NP) Ti–40Nb discs: following plasma treatment, Ti–40Nb discs were passivated by incubation with 0.1 mg ml⁻¹ PLL-g-PEG in HEPES (Surface Solutions, Switzerland) for 30 min at room temperature. Following several rinses with distilled water, the discs were incubated with 25 μM cyclic RGDfK or cyclic RADfK at room temperature for at least 4 h. Prior to cell plating, the discs were washed with sterile phosphate buffered saline (PBS).

2.3. Characterization of the quasi-hexagonal pattern of the gold nanoparticles by scanning electron microscopy
To characterize the quasi-hexagonal gold nanoparticle pattern, the surface of Ti–40Nb discs was examined with SEM (LEO 1530 Gemini, Carl Zeiss), as shown in figure 2. Prior to SEM imaging, carbon was sputtered (MED 020 Coating System Sputter, Bal-Tec, Liechtenstein) on the Ti–40Nb discs to enhance electron scattering, thus enhancing contrast. The interparticle distances as well as the order of the hexagonal patterns were derived by analysing the SEM micrographs (50,000× magnification) with the IMAGEJ plugin ‘Dot analyser’ (kindly provided by Philippe Girard, University of Heidelberg) according to the underlying theory of Kansal et al. [33].

2.4. Auger electron spectroscopy
Sample surfaces were characterized with Auger electron spectroscopy (AES) using a JAMP-9500 F Field Emission Auger Microprobe (Jeol, Japan) with primary electrons of 10 keV at an electron current of 10 nA. For depth profiling, the sample surfaces were sputtered by a scanned beam of 1 keV Ar⁺ ions and spectra were recorded in probe areas of 10 × 10 μm after sputter intervals of 0.3 min.

2.5. Transmission electron microscopy
Cross-sectional transmission electron microscopy (TEM) samples were prepared by focused ion beam (FIB) cutting of lamellae from the near-surface region of the processed Ti–40Nb samples decorated with gold nanoparticles using an FEI Helios nanolab 600i dual beam system (FEI EUROPE, The Netherlands). Prior to the FIB cut, layers of amorphous carbon and platinum were subsequently deposited onto the surface in order to protect the area to be investigated from damages through the 30 kV Ga⁺ ion beam. Structural and local chemical characterization was conducted by conventional and aberration-corrected high-resolution-TEM (HR-TEM, shown in figure 3) using an FEI Tecnai G² 20 microscope (with LaB₆ emitter, scanning unit (STEM), energy dispersive X-ray spectrometer (EDXS), FEI company) and an FEI Titan3 80–300 microscope operated at 300 kV (equipped with a field emission gun, CEOS CetCor C₂-image corrector, high angle annular dark field detector, Gatan Tridimen 863ER for electron energy loss spectroscopy (EELS) and EDXS, FEI company), respectively (figure 4). A.%

2.6. Cell culture and indirect immunofluorescence staining
Human MSCs (hMSCs) derived from bone marrow (Promocell, Heidelberg, Germany) were cultured and plated in proliferation medium including the supplements provided by the manufacturer (Promocell), without antibiotics. Cells from two different donors were investigated (donor1: male, Caucasian, 65 years and donor2: male, Caucasian, 64 years). hMSCs (less than 10,000 cm⁻²) were passivated and biofunctionalized Ti–40Nb discs for 24 h and fixed with 4% (w/v) paraformaldehyde (Sigma-Aldrich, St Louis, USA) in PBS (pH 7.4) for 20 min followed by permeabili-

ization with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS for 5 min. After blocking in 1% (w/v) BSA in PBS, the discs were iculated with anti-vinculin mouse IgG (Sigma-Aldrich) detected by Alexa488 goat anti-mouse IgG (Life Technologies, Carlsbad, USA) to stain FAs and phallolidin-TRITC (Sigma-Aldrich) to label F-actin. The antibodies as well as the phallloidin conjugate were diluted according to the manufacturer’s recommendations. The fluorescently labelled samples were embedded in Mowiol supplemented with 1 μg ml⁻¹ DAPI (Sigma-Aldrich) for nuclear staining and mounted onto a coverslip (standard thickness 1, Carl Roth, Karlsruhe, Germany).

2.7. Fluorescence microscopy and data analysis
Fluorescence imaging was carried out with an Olympus IX inverted microscope (Olympus, Hamburg, Germany) using a Delta Vision RT system (Applied Precision Inc., Issaquah, USA). Here, cells were examined using either a 10× (Neofluor 10×/0.3 phase contrast, Carl Zeiss) air or 60× (PlanApo 60×/1.4, Olympus) oil immersion lens with the resulting pixel sizes of 0.589 and 0.158 μm, respectively. Images were acquired using a cooled CCD camera (Photometrics, Kew, Australia) and processing was controlled by RESOLVE3D (Applied Precision Inc.). All images were analysed with IMAGEn v. 1.43 (https://rsb.info.nih.gov/ij/). Cell area was determined from actin micrographs such that binary images were created by adjusting the threshold individually for each image. If required, neighbouring cells on the same sample were separated manually. Cells were counted and analysed using the analyse particle tool from IMAGEn with a threshold set as 10 μm²—infinity and excluding edges from the analysis to ensure only taking completely displayed cells into account. The area of the cell nuclei was determined similarly by creating binary images. Cell number was determined by counting the nuclei, whereas nucleus area and circularity were determined additionally by using the analyse particle tool with the threshold set to 50–2000 μm². To rule out the effects owing to sample or cell seeding inhomogeneity, 8–10 random images throughout the whole sample areas were acquired from at least three different experiments. Focal adhesion analysis was performed on images acquired with a 60× oil lens as described above. Clusters were outlined and analysed with IMAGEn using the ROI manager and ROI color coder (by Tiago Ferreira) tools. Only clusters larger than 0.5 μm² were considered for analysis.
spindle-shaped cells with a typical area less than 5000 μm² showing different phenotypes. This inherent heterogeneity is especially undesired for further use in regenerative medicine because it accounts for senescent and/or committed cells as mentioned above and evaluated their cell size distribution. As can be clearly seen in figure 1a,b, donor 1 comprises many large cells and only very few small cells (less than 5000 μm²), whereas several small cells are observed in the case of donor 2 (figure 1c,d). These results were also confirmed for cells derived from other frozen stocks of the same donors (data not shown).

### 3. Results and discussion

#### 3.1. Human mesenchymal stem cells on polished Ti–40Nb

Ti–40Nb discs were manufactured by using a melting procedure as described in the experimental section. To demonstrate the heterogeneity of human bone-marrow-derived MSCs (hMSCs) in independent experiments, we seeded cells derived from two different donors of comparable age (donor1, D1: 65 years and donor2, D2: 64 years) on polished non-patterned Ti–40Nb surfaces (figure 1a,b) and Ti–40Nb surfaces (figure 1c,d). Note the typical heterogeneous mixture of cells, showing different phenotypes. This inherent heterogeneity becomes particularly apparent for cell size, ranging from small spindle-shaped cells with a typical area less than 5000 μm² to up to 30000 μm² for large spread cells. The spindle-shaped cells have a fibroblastic-like morphology, whereas the large cells are spread, and/or irregular shaped and very flat with the exception of the peri-nuclear region. The former type resembles fibroblast morphology; it should be noted however that these cells cannot be considered as fibroblasts, thus differing in their function of secreting and assembling ECM. The latter type is especially undesired for further use in regenerative medicine because it accounts for senescent and/or committed cells [34–36]. A cell size below 1000 μm² mostly indicates poorly attached or dividing cells.

Besides the inherent heterogeneity of the hMSC phenotype, an additional challenge is the diversity between different donors. We chose hMSCs from two donors as mentioned above and evaluated their cell size distribution. As can be clearly seen in figure 1a,b, donor 1 comprises many large cells and only very few small cells (less than 5000 μm²), whereas several small cells are observed in the case of donor 2 (figure 1c,d). These results were also confirmed for cells derived from other frozen stocks of the same donors (data not shown).

#### 3.2. Characterization of gold nanoparticles on Ti–40Nb discs

Kilian et al. [37] reported that cell shape and contractility influence lineage commitment of bone-marrow-derived hMSCs. Therefore, a defined cell microenvironment, in particular for its physical and chemical features, might represent a successful approach to control hMSC anchorage to the implant material as well as to direct cell responses. The defined deposition of gold nanoparticles on surfaces is an elegant way to spatially control and direct cell adhesion through topography or by functionalizing the gold nanoparticles with adhesion-promoting ligands. Here, we used polished Ti–40Nb alloy surfaces for nanopatterning and subsequent biofunctionalization. To achieve a controlled surface topography, a hexagonally ordered pattern of gold nanoparticles was created on the surface by a dip-coating technique based on self-assembly of diblock copolymer micelles (BCMN). The scheme in figure 2 depicts the surface treatment and BCMN process. Here, we chose the copolymers such that with a constant retraction velocity of 25 mm min⁻¹ average interparticle distance of 68 ± 13 nm (order 0.54) for smaller spacing or 96 ± 17 nm (order 0.56) for larger spacing can be obtained. The order of the hexagonal gold nanoparticle pattern as well as the interparticle distances was determined by evaluation of SEM images (figure 2b,c) as described in the experimental section. For cell experiments, a distance of approximately 68 nm was chosen, being the threshold for integrin lateral clustering and assembly of stable FAs in cells of mesenchymal origin [38,39].

AES (see electronic supplementary material, figure S1) was used to chemically characterize the surface state. Survey spectra of the outermost surface reveal the main presence of Ti- and Nb-oxide species. Local AES analyses at selected points (covering probe areas of about 50 nm), which are visible in the SEM images in figure 2 as white contrast, clearly confirm that these are indeed gold particles. Upon depth profiling, Au was detected up to a sputter depth of approximately 5 nm. However, the main characteristics of the depth profile indicate the presence of a Ti- and Nb-oxide layer with a thickness of approximately 18 nm. This oxide layer on Ti–40Nb generated by the above-described process is much thicker than the natural one with only less than 5 nm [9]. Altogether, the AES results demonstrate the presence of nanoscale Au species above a (Ti,Nb)-oxide layer. However, being the characterization of the Au species at the lower detection limit of the method, it is necessary to apply analyses at a higher resolution level.

TEM investigations of cross-sectional areas of Ti–40Nb samples confirm the presence of an oxide layer at the sample surface (figure 3). This layer has a thickness of at least 10 nm and is evidenced by the darker zone at the sample surface in
the scanning TEM (STEM) images displayed in figures 3b and 4b. Here, the reduction of the average atomic number owing to the incorporation of light O atoms effectively reduces the STEM contrast. Evidence for this surface oxidation arises from EELS measurements shown in figure 4. EEL spectra were collected in STEM mode at positions 1–10 along the yellow line across the oxide–metal interface as indicated in the STEM image in figure 4b. Here, the dispersion and energy range of the EEL spectrometer were adjusted to allow for a simultaneous acquisition of losses at the Nb–M5,4, Ti–L3,2 and O–K absorption edges, respectively. The relative amounts of Nb, Ti and O were determined from an evaluation of the near edge absorption intensities after appropriate background subtraction (see dashed line in figure 4c). The resulting concentration profiles across the interface are displayed in figure 4a. The measurements clearly evidence the enrichment of oxygen within the surface layer. Detailed analyses of the absorption edges further reveal a small chemical shift of the Ti–L3,2
size gold nanoparticles. Here, we use gold nanoparticles of approximately 5 nm in size to investigate their influence on hMSC heterogeneity, in particular on cell size. In contrast to the aforementioned works dealing with topographical investigations for several days, we studied early cell–surface interactions after 24 h.

3.3. Human mesenchymal stem cells on nanopatterned Ti–40Nb alloys

To determine the effects of surface topography on cell phenotype, surfaces were modified by deposition of gold nanoparticles on Ti–40Nb via BCMN. Here, hMSCs were seeded on polished as well as on NP discs for 24 h and heterogeneity of the population was analysed, in particular with respect to the number and fraction of large-sized cells. Cells plated on patterned Ti–40Nb adhere and spread similarly to cells grown on non-patterned Ti–40Nb alloys and control (figure 3a–c). However, on patterned surfaces the overall cell number (figure 5g) as well as cell confluence (data shown in the electronic supplementary material) is reduced. The absolute numbers of large-sized (more than 10,000 μm²) cells are comparable in all three conditions: control surfaces, non-patterned and NP Ti–40Nb alloys (data not shown). Hence, modifying the surface topography by the deposition of nanoparticles of approximately 5 nm could not improve the ratio of fibroblast-like cells nor resulted in a decreased number of large cells. In this study, we analysed short-term effects induced by nanoscale surfaces to reduce heterogeneity within a cell population and among donors. Dalby and co-workers [41,42], for example, investigated the influence of nanoscale topographies on long-term maintenance of the stem cell phenotype or the induction of osteogenesis. Also, migration of osteoblasts was found to be influenced by topographical modifications on the nanoscale [43]. Further studies will be necessary to determine the influence of nanoscale topographies on long-term cell behaviour, and, in turn, the surface modifications after cell matrix deposition.

Besides their effects on surface topography, nanoparticles arranged in patterns are an elegant tool for controlling cell adhesion via immobilization of specific thiolated biomolecules and for confining cell–surface interaction to defined sites on the surface. Passivation of the surface between the particles is crucial to assign the observed effects to the immobilized ligands only. To obtain a passivation of the space between the nanoparticles, a polyethylene glycol (PEG) layer was physiosorbed on the surface, thereby restricting cell–substrate interactions only at the particles. As expected, adhesion of hMSCs after 24 h on these passivated surfaces is very poor and spread cells are absent (figure 5d).

To specifically design the cell–surface interface, directly after PEG-passivation the gold nanoparticles were functionalized with adhesion-promoting peptides. Here, we immobilized either the thiolated, cyclic pentapeptide cRGDFK (cyclo-Arg-Gly-Asp-D-Phe-Lys) or the control peptide cRADfK (cyclo-Arg-Ala-Asp-D-Phe-Lys) on the gold nanoparticles. Thus, cells seeded on these surfaces are only able to adhere to the surface through the presented immobilized ligands. Figure 5e,f presents overview images of hMSCs on both ligands after 24 h. This time point was chosen to allow cells to adhere and spread properly, on the one hand. On the other hand, this short-term incubation ensured that the observed effects only stem from the functionalized surfaces and no significant cell-induced alterations of the

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**Figure 4.** EEL spectra as collected from the subsurface positions 1 and 2 indicated in the STEM image in (b). The displayed range of loss energies covers the Ti–L₃,₂ and O–K absorption edges. A small chemical shift between the two Ti–L₃,₂ edges is highlighted by a dashed vertical line.

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edges upon crossing the oxide–metal interface which is indicative of an oxidation of Ti within the oxide layer (not shown here). HR-TEM images further demonstrate that the surface oxide is amorphous, as no indications of crystalline structures were ever observed (also not shown).

Both the TEM and STEM images in figure 3a,b display nanoparticles whose strong contrast is already indicative of the high atomic number of the material they are comprised. There are large particles with sizes of 6–7 nm and a number of smaller particles with diameters in the range of 2–3 nm. In similar micrographs (as the one displayed in the TEM image in figure 3a) from other cross-sectional areas of the sample, we could detect a comparable number of Au particles which is indicative of a homogeneous (areal) particle density across the whole sample. HR-TEM images of the particles confirm that the particles are indeed gold. The examples presented in figure 3c for a large particle and figure 3e for a small particle, and their diffractograms as obtained by fast Fourier transformation (FFT) clearly show the crystal structure of face-centred cubic (fcc) Au as seen along (110) zone axes.

In the past, several groups investigated the topographical influence on a variety of cellular functions. It has been shown that fibroblasts sense changes in the surface roughness of approximately 10 nm, which affects their proliferation and spreading [40]. Sjöström *et al.* [32] described an increase in MSC osteogenic potential on 15 nm compared with 8 nm...
substrate has occurred yet. Owing to their inability to trigger cell-spreading, data of NP, PEG-passivated surfaces (shown in figure 5d) are not considered in the following analysis. As shown in figure 5g, the overall number of cells adhering to cRGDfK-functionalized substrates is comparable to NP (without passivation) surfaces, whereas fewer cells adhere on cRADfK.

Considering the size distribution in more detail, a clear effect can be seen on both functionalized surfaces. Figure 5h displays the percentage of fibroblast-like (less than 5000 \( \mu \text{m}^2 \)), medium-sized (5000–10 000 \( \mu \text{m}^2 \)) and large (more than 10 000 \( \mu \text{m}^2 \)) cells from both donors. Compared with glass (control) and non-functionalized NP Ti–40Nb surfaces, the fraction of fibroblast-like cells is enhanced on both functionalized NP alloys to approximately 80%, while medium-sized and large cells were decreased to about 10% and 5%, respectively. At the same time, the variability between the donors is strongly decreased, rendering these functionalized surfaces a promising tool for selecting MSCs with homogeneous phenotype. The two used ligands cRADfK and cRGDfK differ in their specificity and affinity for cell surface receptors, namely integrins. In these experiments, the observed size selection appears to be independent of integrin-specificity of the presented ligand (cRGDfK or cRADfK), but rather of the arrangement and availability of cell anchorage sites. However, cells adhering to cRADfK are slightly reduced in overall cell number; additionally, these cells spread less (figure 5e,f) compared with cells on cRGDfK. Cell structures at the interface with materials, especially FAs, are crucial for the transduction of forces from the surface to

**Figure 5.** Cell adhesion and cell size distribution of hMSCs adhering to different surfaces after 24 h. Fluorescent micrographs of hMSC populations on (a) control, (b) polished non-patterned Ti–40Nb, (c) NP Ti–40Nb, (d) NP and PEG-passivated Ti–40Nb surfaces, (e) NP, PEG-passivated cRGDfK-functionalized Ti–40Nb surfaces and (f) NP, PEG-passivated cRADfK functionalized Ti–40Nb surfaces (F-actin: red, DAPI: green). The box plot in (g) depicts the cell number per acquired image. The chart in (h) displays the cell size distribution of both donors for the surface conditions shown in (a,c,e,f). Note the increase in fibroblast-like cells (1000–5000 \( \mu \text{m}^2 \)) on the functionalized NP surfaces. *\( p = 0.0001.\)
the actin cytoskeleton and vice versa. Kilian et al. [37] used shape confinement at the microscale to study cell tension and found that increased acto-myosin contractility promotes osteogenesis in MSCs. Here, we confined not the cell shape but rather their anchorage by patterns consisting of single nanoparticles. Thus, the adhering cells can adopt any shape, as long as they adapt to the substrate by establishing new FAs. Furthermore, adhesion signalling might be involved in the regulation of cell senescence, as it has been shown for example, for paxillin and c-Src as well as cytoskeletal proteins [44]. It remains however unclear, if the reduced expression in key adhesion molecules during cell senescence could modulate cell ability to adhere and adapt to different materials because of altered protein turnover in FAs.

3.4. Focal adhesions on nanopatterned alloys

hMSCs were seeded on Ti–40Nb NP with either cRGDfK or cRADfK ligands as described above. Twenty-four hours after cell seeding, FAs were identified in fixed cells by indirect immunofluorescent staining of vinculin and visualized by fluorescence microscopy. Both on polished and on NP non-passivated Ti–40Nb discs, hMSCs show robust peripheral FAs (figure 6c,d). Cells grown on Ti–40Nb discs passivated and patterned with cRADfK-decorated nanoparticles, display smaller and undefined vinculin clusters which are not localized at the cell periphery, whereas cells adhering to cRGDfK exhibit elongated and defined FAs at the cell margin (figure 6c,d). Note that the number of FAs per cell is reduced on all NP samples in comparison with the non-patterned surface as
shown in figure 6c. However, offering cRGDK increases the number of FAs per cell on these substrates dramatically. These results indicate that the reduced cell size observed on samples functionalized with the cRADfK ligand is owing to poor adhesion and lack of forming a sufficient number of mature FAs.

For the use of biological material in regenerative medicine, it is important to guarantee a high reproducibility and homogeneity between different batches on the one hand, and predictable and/or uniform samples, on the other hand. To gain information about surface uniformity, cell coverage (surface area covered by cells) on the different coatings was analysed (data displayed as box plots in the electronic supplementary material, figure S2). On control and polished, untreated Ti–40Nb surfaces, the cell coverage is extremely variable for different areas analysed, evidencing inhomogeneous cell settling throughout the samples. This variability is reduced on the nanostructured surfaces, even on the non-functionalized nanoparticles.

Our observations that hMSCs form large vinculin clusters on untreated and on non-functionalized Ti–40Nb discs is in agreement with the recent findings of Sjöström et al. [32]. The presence of nanotopographies owing to nanoparticles of 5–10 nm size might therefore promote on the long-term osteogenic differentiation of hMSC via the local enhancement of FA maturation. Regarding the use of RGD motifs covalently attached to biometals, Jäger et al. [45] showed that hMSC osteogenic stimulation is not significantly improved in the presence of these peptides. This report further supports our findings that RGD motifs promote adhesion of hMSC and the formation of mature FA, while the cells still exhibit their typical non-committed stem cell phenotype.

4. Conclusion

Using Ti–40Nb alloys, which present the advantage of closely matching the elastic modulus of bone, we probed the response of hMSCs to surface nanotexturing and further functionalization with cRGDK peptides. We determined the effects of these surface modifications on the settling of hMSC having heterogeneous phenotype and on adhesion. This shows a proof-of-principle for the use of such materials for the selection of hMSC subpopulations and decreasing donor variability. The results shown here suggest that by combining spatial cues with specific adhesive cues at the nanoscale, hMSC adhesion is optimized while maintaining the typical phenotype of non-committed stem cells. Studies with such materials could test in the future whether these cells would show the same differentiation potential and whether the expression of specific senescence markers is abolished.

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